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"Ação do Mastoparano *Polybia*-MPII nas fibras musculares e na junção neuromuscular: Um estudo morfológico, imunohistoquímico e biofísico"

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Biologia Celular.

Orientadora: Profa. Dra. Maria Alice da Cruz Höfling

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Sonda-me

Aline Barros

Sonda-me, Senhor, e me conhece, quebranta o meu coração
Transforma-me conforme a Tua palavra
E enche-me até que em mim se ache só a Ti
Então, usa-me, Senhor, usa-me

Como um farol que brilha à noite
Como ponte sobre as águas
Como abrigo no deserto
Como flecha que acerta o alvo

Eu quero ser usado, da maneira que Te agrade
Em qualquer hora e em qualquer lugar, eis aqui a minha vida
Usa-me, Senhor, usa-me

Sonda-me, quebranta-me
Transforma-me, enche-me, e usa-me, Senhor.

Índice

Resumo

Venenos e toxinas de animais peçonhentos são importantes ferramentas farmacológicas para o estudo de fenômenos biológicos. Os venenos produzidos por insetos sociais da ordem Hymenoptera têm chamado a atenção de bioquímicos, imunologistas, farmacologistas e neurologistas, tanto do ponto de vista clínico como biotecnológico. Neste trabalho o mastoparano (MP) Polybia-MPII (INWLKLGKMVIDAL-NH_2), do veneno da vespa *Polybia paulista*, serviu de ferramenta para avaliar se o peptídeo tem ação miotóxica e neurotóxica, bem como determinar a natureza dessa ação em biomembranas. A ação miotóxica foi avaliada pela análise morfológica ao microscópio de luz e eletrônico e morfometria durante as fases degenerativa (3 e 24 horas) e regenerativa (3, 7 e 21 dias) após a injeção intramuscular de 0,25 μg/μl do peptídeo. Com o objetivo de identificar os eventos celulares e moleculares que acompanham essas alterações nos diferentes períodos analisados, foi avaliada a expressão das citocinas pró-inflamatórias, o fator de necrose tumoral (TNF-α) e o interferon gama (IFN-γ). Em geral, na avaliação da ação local de venenos e toxinas, tem sido dado ênfase à capacidade de induzir mionecrose e tem-se negligenciado a capacidade de induzir apoptose por parte dessas substâncias. No presente trabalho, através da técnica de TUNEL e da imunomarcação de caspases 3 e 9, foi analisado a capacidade do Polybia-MPII em promover morte celular programada por apoptose no músculo tibial anterior. A ação neurotóxica do Polybia-MPII foi avaliada por microscopia confocal e microscopia eletrônica de transmissão e consistiu em investigar a organização da inervação dos terminais em secções longitudinais através da marcação com alfa-bungarotoxina conjugada com tetrametilrodamina (TRITC-αBTX) ou com isotiocianato de fluoresceína (FITC-αBTX), e contra-imunomarcados com sinaptofisina (marcador específico para vesículas sinápticas) e neurofilamentos, e pela contagem de vesículas sinápticas nos terminais colinérgicos. Os resultados mostraram pela primeira vez que o mastoparano de *P. paulista* é um potente indutor de mionecrose e apoptose. A atividade miotóxica do peptídeo mostrou iniciar-se pela lise do sarcolema seguida por diferentes estados patológicos comprometendo a organização miofibrilar, seguida pela lise das miofibras e das miofibrilas, com preservação da lâmina basal. A indução de apoptose pelo peptídeo foi demonstrada morfologicamente pela técnica de TUNEL, presença de caspase 3 e 9 e pela severa alteração mitocondrial e alteração da cromatina dos núcleos

observadas por microscopia eletrônica, as quais coincidiram com expressão aumentada de IFN-γ e TNF-α, esta uma citocina que medeia a ativação de uma das via das caspases que leva à morte celular programada por apoptose. Com relação à atividade neurotóxica, os resultados mostraram que o Polybia-MPII possui ação pré-sináptica, devido à significativa diminuição no conteúdo de vesículas sinápticas nos terminais pré-sinápticos, corroborando com dados do nosso grupo que evidenciaram através de estudos eletrofisiológicos a mesma ação neurotóxica pré-sináptica causada pelo veneno bruto de *P. paulista*. Esses efeitos foram variáveis ao longo dos diferentes períodos analisados, mostrando que as alterações eram transitórias e que os fenômenos regenerativos permitiam o restabelecimento da morfologia original. Os estudos biofísicos feitos em membranas artificiais mostraram que o Polybia-MPII interage com a bicamada lipídica da membrana, provavelmente utilizando o triptofano como ancoragem através de um mecanismo conhecido como "carpet", desestabilizando o equilíbrio iônico e promovendo a ruptura da membrana. Os estudos biofísicos mostraram-se consistentes com as observações morfológicas ao microscópio de luz e eletrônico. Os dados permitem concluir que o mastoparano Polybia-MPII é um potente indutor de mionecrose em músculo esquelético e sua atividade neurotóxica periférica pode ser qualificada como moderada e pré-sináptica; além de induzir apoptose em fibra muscular, cujo significado precisa ser investigado. A reversibilidade dos efeitos causados pelo mastoparano está de acordo com os dados clínicos observados em acidentes com ferroadas de *P. paulista* em indivíduos não alérgicos, os quais se caracterizam na maioria por serem de pouca gravidade, curta duração e auto-limitantes.

Abstract

Venoms and toxins of poisonous animals are important pharmacological tools to study biological phenomena. The venom of social insects as those produced by Hymenoptera get attention from biochemistries, immunologists, biologists, pharmacologists and neurologists, as in a clinic view as biotechnologically. In this study the mastoparan (MP) Polybia-MPII, (INWLKLGKMVIDAL-NH_2) from *Polybia paulista* wasp venom, was used as a tool to analyze if the peptide has myotoxic and neurotoxic effects, as well to determine its action on biomembranes. The myotoxicity of the mastoparan was evaluated during the degenerative phase (3 and 24 hours) and regenerative phase (3, 7 and 21 days) after the intramuscular injection of 0.25 μg/μl of the peptide using light microscopy, transmission electron microscopy and morphometry. With the purpose of determining the cellular and molecular events accompanying the alterations caused by *P. paulista* wasp mastoparan in these time-points the expression of proinflammatory cytokines, such as the tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) were evaluated. In general, studies on the local effects of venom and toxins have focused on their myotoxic potential, whereas the potential to induce apoptosis has been neglected. In this work, the ability of Polybia-MPII in inducing cell death by apoptosis on the tibial anterior muscle was assessed by TUNEL technique and immunohistochemistry for caspase 3 and caspase 9. The neurotoxic action of Polybia-MPII was evaluated by examining the organization of terminals innervation in longitudinal sections labelled with tetramethylrhodamine-conjugated- or isothiocyanate fluorescein-conjugated-alpha-bungarotoxin (TRITC or FITC α-BTX receptor, respectively) and counter labelled with a combination of anti-synaptophysin (a specific marker to synaptic vesicles) and anti-neurofilament protein using confocal microscopy and transmission electron microscopy, and by counting the synaptic vesicle within the cholinergic terminals. The results showed for the first time that the Polybia-MPII is a potent inducer of myonecrosis and apoptosis. The myotoxic action of the peptide initiated with the lyses of the sarcolemma followed by the development of different pathologic stages affecting myofibrillar organization, and eventually myofibrils and myofibre lyses, but maintaining unaffected the basal lamina. The ability of the peptide to induce apoptosis was demonstrated by TUNEL technique, immunolabelling of caspase 3 and 9, severe mitochondrial damage and alteration of the structure of nuclei chromatin seen

by electron microscopy. Such effects were positively correlated with increased IFN-γ and TNF-α expression, being the last one a cytokine which mediates the caspase pathway leading to programmed cell death by apoptosis. Concerning Polybia-MPII neurotoxicity, the results showed a pre-synaptic action of the peptide which was inferred by a decrease in synaptic vesicles content of pre-synaptic terminals, in agreement with electrophysiological studies done by our group with the *P. paulista* crude venom. These alterations varied along the time intervals analyzed, evidencing that the alterations were transitory and the regenerative phenomena allow to morphological reestablishment. The biophysical studies performed on artificial membranes, showed that the peptide interacted with the lipid bilayer probably anchored by tryptophan and by a mechanism known as carpet. As a result, the ionic balance is impaired inducing membrane leakage. The biophysical data were consistent with the morphological observations by light and electron microscopy. Data allow concluding that Polybia-MPII mastoparan is a mighty inducer of myonecrosis whereas the peripheral neurotoxicity could be considered as moderate and pres-synaptic. Besides, the peptide has an apoptotic-inducing potential on skeletal muscle fibre, the meaning of which deserves further investigation. The reversibility of the mastoparan effects is in accordance with clinical data in non-allergic patients stung by *P. paulista*, whose outcome is considered of mild, short-lived and self-limiting in severity.

Introdução

A ordem Hymenoptera é composta por vespas, abelhas e formigas, e representa o maior grupo dentro da Classe Insecta. Os venenos de Hymenoptera apresentam diferentes compostos farmacologicamente ativos, como enzimas (fosfolipases e hialuronidase), peptídeos (melitina, apamina, mastoparano, bombolitinas) e compostos de baixa massa molecular (aminas biogênicas, tais como, histamina, serotonina, acetilcolina, poliaminas, além de carboidratos e lipídeos).

Essa ordem é responsável por 9,3% a 28,5% dos casos de sensibilização na população mundial (Antonicelli et al, 2002), sendo os relatos clínicos sôbre acidentes mais freqüentes com abelhas. Dentre os Hymenoptera, as vespas são os insetos que apresentam maior importância médica, principalmente em indivíduos que sofreram múltiplas ferroadas e/ou apresentam reação sistêmica como o choque anafilático. Entretanto, a grande maioria das manifestações clínicas resume-se a uma reação cutânea imediata, não alérgica, confinada ao local da ferroada e de duração breve, que se caracteriza por leve edema, hiperemia, urticária e pequeno aumento da temperatura no sítio afetado e que, em alguns casos, pode passar despercebida ou ser sentida como um leve ardume (Hamilton et al, 2001).

Nos casos de reações alérgicas decorrentes de múltiplas ferroadas, o edema local pode evoluir para corpos de granulação endurecidos e pruriginosos, palpáveis através da pele. Nestes casos, as múltiplas pápulas ou pústulas podem ser agravadas por infecções bacterianas secundárias (Parrino et al, 1981; deShazo et al, 1990; Janniger et al, 1994; Reisman, 1994; Hamilton et al, 2001). Nos casos graves, envolvendo reações sistêmicas de sensibilização, os indivíduos podem sofrer choque anafilático, urticária generalizada, broncoespasmo, hipotensão, colapso cardiovascular e perda de consciência, além de vômito, diarréia, dor de cabeça, convulsão e/ou confusão mental (Schmidt, 1986; Tamir et al, 1996). Embora raro, há indivíduos que desenvolvem isquemia cardíaca, encefalomielite e outros sintomas graves (Boz et al, 2003). Possivelmente a maioria das reações anafiláticas são mediadas por imunoglobulinas de classe E (IgE), porém as reações alérgicas podem também ser de base celular, ou ambas as mediações podem estar envolvidas (Schmidt, 1986).

Ao nível celular, os venenos de vespas podem causar citólise, hemólise e quimiotaxia de macrófagos e leucócitos polimorfonucleares (Habermann, 1972), e degranulação de mastócitos (Nakajima, 1986).

Estudos sobre a ação dos venenos de Hymenoptera têm sido realizados *in vitro*, em sinaptossomas ou fatias de córtex cerebral e hipocampo, e versam sobre a liberação de neurotransmissores, envolvimento dos compostos nos canais iônicos; resultando na liberação de cálcio intracelular (Rivers et al, 2005) e sódio, promovendo a facilitação excitatória e inibitória da transmissão sináptica (Sahara et al, 2000); e atividades nos receptores nicotínicos, GABA e L-glutamato (Harsch et al, 1998; Pizzo et al 2000; 2004; Sahara et al, 2000). Já os estudos relativos aos efeitos neurotóxicos periféricos utilizam preparações neuromusculares isoladas e investigam a força de contração muscular e potenciais elétricos da junção neuromuscular (Paes-Oliveira et al, 1998; Ferber et al, 1999; 2000; Sahara et al, 2000).

O conhecimento da especificidade das toxinas presentes nos venenos de vespas e outros Hymenoptera, bem como a sua estrutura molecular, têm importância fisiológica, farmacológica e bioquímica, e faz-se necessário para que terapias adequadas possam ser utilizadas na neutralização das reações nas vítimas. Além de sua relevância para a saúde pública, podem servir de alvo para indústria farmacêutica na obtenção de novos fármacos e para fins de controle de pragas na agricultura (Blagbrough et al, 1992).

O veneno das vespas

Dentre os componentes do veneno de vespas, destacam-se as fosfolipases, as aminas biogênicas e os mastoparanos.

As fosfolipases são proteínas que, embora em pequena quantidade, representam os componentes alergênicos dos venenos de vespas sociais. Devido à sua participação na regulação do metabolismo de fosfolipídios em biomembranas, a fosfolipase presente no veneno de vespas também pode atuar na ruptura de membranas celulares (Rivers et al, 2005), conduzindo à formação de poros e por conseqüência à lise celular, como observado para fosfolipases provenientes do veneno de abelhas (Dotimas et al, 1987; Murakami e Kudo, 2002). Tal permeabilização promove a inserção dos antígenos na célula,

desencadeando severas reações alérgicas e/ou inflamatórias (Nakajima, 1986). Sua ação hidrolítica, dependente de íons Ca^{2+}, é potencializada pela atividade das lipases, esterases, fosfatases presentes no veneno e fosfolipases endógenas. As fosfolipases agem sinergicamente com outras toxinas, causando lise de eritrócitos e mastócitos e ocasionando também inibição da coagulação sanguínea (Palma e de Souza, 2008).

Estudos experimentais têm demonstrado que as fosfolipases encontradas em venenos de vespas aumentam a permeabilidade da membrana plasmática ao Na^{+} (Sahara et al, 2000), resultando em edema e morte celular por oncose (Rivers et al, 2002), um tipo de necrose. Além de causar hipotensão e aumento da permeabilidade vascular, podem provocar contrações musculares e parada respiratória. A ação da fosfolipase A_2 também tem sido relacionada à regulação da apoptose, com envolvimento em mudanças no metabolismo de glicerolipídeos (Taketo e Sonoshita, 2002). Ativadores de fosfolipase foram descritos como indutores de exocitose dos grânulos das células da adenohipófise de ratos, ação esta partilhada pela melitina (abelhas) e pelo mastoparano (vespas) (Kuhihara et al, 1986).

Algumas acil-poliaminas, como a philanthotoxina (PhTX) do veneno da vespa solitária *Philantus triangulum* (Hymenoptera, Crabonidae), são potentes neurotoxinas capazes de inibir os receptores de glutamato de músculo de outros insetos, através do bloqueio de canais iônicos, principalmente os de Ca^{2+}, levando a presa à paralisia (Blagbrough et al, 1992). As propriedades farmacológicas das acil-poliaminas têm sido vistas como ferramentas potenciais para desenvolvimento de bioinseticidas e para o estudo da fisiologia dos canais iônicos, dos receptores dos neurotransmissores, e de moléculas envolvidas no transporte axonal. Além disso, podem ser de alta relevância para o estudo de doenças como Alzheimer, epilepsia, dentre outras relacionadas à disfunção dos sistemas glutamatérgico e gabaérgico, uma vez que possuem ação sôbre os receptores desses neurotransmissores (Sahara et al, 2000; Pizzo et al, 2004).

Estudos experimentais atribuem aos peptídeos dos venenos de vespas envolvimento em processos inflamatórios desencadeados pelas ferroadas, na lise da membrana celular, liberação de histamina e conseqüente vasodilatação, aumento da quimiotaxia de neutrófilos e linfócitos (Nakajima et al, 1986; Ho et al, 1998; Mendes et al, 2004). Dentre os peptídeos identificados e caracterizados nos venenos de Vespidae destacam-se os mastoparanos.

Os mastoparanos (MP), assim como a melitina do veneno de *Apis mellifera*, são tetradecapeptídeos policatiônicos, anfifílicos, que apresentam intensa atividade hemolítica, além de atuarem na degranulação de mastócitos e na quimiotaxia de leucócitos polimorfonucleares (de Souza et al, 2004). Uma atividade importante demonstrada para o MP é a permeabilização da membrana mitocondrial interna, induzindo a abertura de poros proteináceos (Pfeiffer et al, 1995; Curtis e Wolpert, 2002). Tal fenômeno está associado à morte celular por apoptose e por necrose, ambos relacionados com sobrecarga de Ca^{2+} na mitocôndria (Javadov e Karmazyn, 2007).

Uma das características mais conhecidas dos MPs é a sua ação na liberação/exocitose de grânulos e/ou vesículas secretoras, como histamina, catecolaminas, prolactina e serotonina (Kurihara et al, 1986; Katsu et al, 1990). Nas ilhotas pancreáticas, o MP estimula a liberação de glucagon e insulina através de proteínas reguladoras ligadas à guanosina trifosfato (GTP) (proteína G) e acopladas à ativação das fosfolipases A e C, e à liberação de Ca^{2+} (Komatsu et al, 1992). Em cardiomiócitos atriais, o MP induz a liberação dos grânulos que contém o fator natriurético atrial (Bensimon et al, 2004). Todas essas ações sugerem que o MP age na maquinaria de exocitose celular (Vitale et al, 1994).

Ação do veneno de *Polybia paulista* (Paulistinha)

A vespa *Polybia paulista* (Vespidae, Polistinae) (Figura 1), endêmica do sul do Brasil e considerada uma das espécies mais agressivas da tribo Epiponini, é responsável pela maioria dos acidentes com vespas no estado de São Paulo (Oliveira e Palma, 1998).

O veneno bruto de *P. paulista* é constituído por 30% de mastoparanos (Palma, informação verbal).

Estudos experimentais em preparações nervo frênico-diafragma de camundongos submetidos à estimulação elétrica indireta mostraram que o veneno é miotóxico, pois induz nas fibras musculares lesões do tipo delta, indicativas de lise do sarcolema, hipercontração das miofibrilas e miólise da fibra, além de alterações mitocondriais e nucleares, cujas características sugerem a ocorrência de fenômenos apoptóticos (Paes-Oliveira et al, 1998; Oliveira, 2000; Paes-Oliveira et al, 2000). Estudos eletrofisiológicos indicaram que o veneno bruto de *P. paulista* tem atividade neurotóxica atuando pré- e pós-sinápticamente na placa motora, e levando ao bloqueio da transmissão neuromuscular. Além de induzir a

depleção de vesículas sinápticas, causa 60% de bloqueio da resposta contraturante à acetilcolina (Paes-Oliveira et al, 1998; 2000). *In vivo*, a injeção intraperitonial do veneno bruto de *P. paulista* induz ao recrutamento dose- e tempo-dependente de neutrófilos, eosinófilos e leucócitos mononucleares, e o extravasamento de leucotrienos na cavidade peritoneal (de Paula et al, 2006).

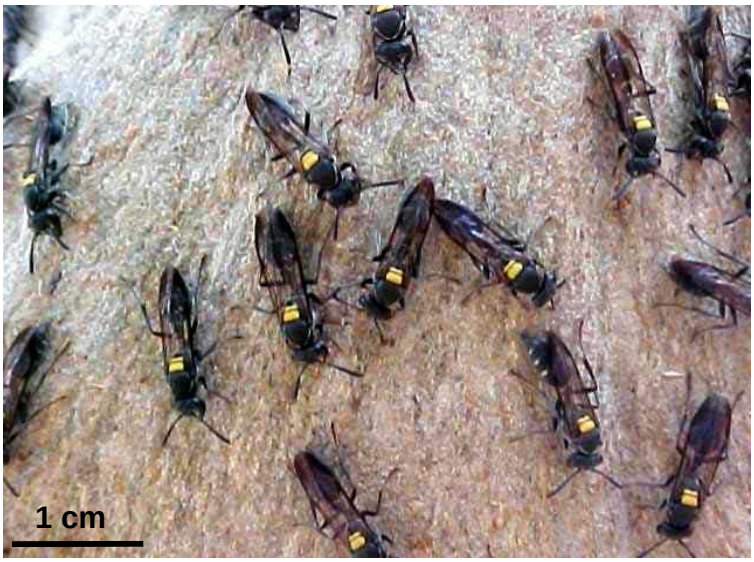

Figura 1: Detalhe de indivíduos adultos de *P. paulista* sobre o ninho. (Thalita Rocha, Fábio Barros Britto, Thiago A. O. Pietrobon)

Morte celular por mionecrose

A patogênese da mionecrose tende a seguir um padrão, independentemente de ter sido desencadeada por toxinas hemorrágicas ou miolíticas (Ownby e Colberg, 1988; Prianti Jr. et al, 2003; Toyama et al, 2003; Beghini et al, 2004; Rodrigues-Simioni et al, 2004), ou outro agente miotóxico. Inicialmente, a mionecrose ocorre pela permeabilização ou lise do sarcolema, e em seguida pelo desequilíbrio iônico, aumento do influxo de Ca^{2+}, distúrbios osmóticos e edema celular. A lise do sarcolema, o influxo de Ca^{2+} promove a formação de lesão delta, hipercontração das miofibrilas, seguida pela perda de registro dos sarcômeros, aglutinação compacta dos miofilamentos e o desarranjo do retículo sarcoplasmático e das mitocôndrias (Serafim et al, 2002; Harris et al, 2003; Reali et al, 2003).

Sabe-se que o dano muscular acarreta ativação de diferentes populações de células inflamatórias responsáveis por uma maior produção de citocinas inflamatórias, remoção de "debris" necróticos e, por fim, reparo e regeneração tecidual (Tidball, 1995). As citocinas pró- e anti-inflamatórias produzidas por células inflamatórias e pelas próprias fibras musculares vão influenciar o processo de degeneração e regeneração do tecido muscular (Petricevich, 2006), e podem estar relacionadas aos eventos pró-apoptóticos mediados por caspase (Sonnet et al, 2006) e processos de eliminação de células por necrose.

A apoptose em músculo estriado esquelético pode ser resultado de condições adversas, como atrofia e distrofia muscular (Sandri et al, 1995, 1998; Tews e Goebel, 1997), ruptura na miofibrila por exercício físico, caquexia (Hasselgren e Fischer, 2001), isquemia, ou outros agentes endógenos e exógenos (Yasuhara et al, 2000). Entretanto, uma vez que a fibra muscular é um sincício e pode haver mais de 100 núcleos/fibra, não se sabe, até o presente, qual é a conseqüência da perda controlada de alguns dos mionúcleos para a fisiologia do músculo, uma vez que em tais processos ocorrem sem que haja a completa destruição da célula (Hilder et al, 2005).

Os poros formados na membrana mitocondrial interna, por diversos fatores, incluindo alteração da concentração iônica celular (Sokolove e Kinnally, 1996; Berman et al, 2000; Belizário et al, 2007), e a sobrecarga de Ca^{2+} podem levar à formação de edema mitocondrial e à liberação de proteínas mitocondriais, como o citocromo c (Raff, 1998), que uma vez no citoplasma ativa a pró-caspase-9, desencadeando uma cascata de eventos que, conduzirá a apoptose; condensação e fragmentação do DNA, e produção de corpos apoptóticos (Casciola-Rosen et al, 1996; Green e Reed, 1998; Dalla Libera et al, 1999; Hilder et al, 2005).

No músculo estriado esquelético, a ativação da caspase 3 induz a proteólise das proteínas contráteis actina e miosina (Du et al, 2004). Evidências comprovam que a ativação da caspase no músculo estriado esquelético ocorre após 12 horas da aplicação de endotoxinas (Supinski e Callahan, 2006; Sonnet et al, 2006).

Sabe-se que a ativação das caspases é uma das vias responsáveis pela iniciação e progresso da apoptose (Hilder et al, 2005); sendo a caspase 3 responsável pela formação dos corpos apoptóticos (Dalla Libera et al, 1999). Esta forma de morte celular é morfologicamente distinta da necrose e ocorre sem que haja um processo inflamatório

(Casciola-Rosen, 1996; Dalla Libera et al, 1999). Em ambos os processos as alterações nas fibras musculares esqueléticas afetam a estrutura original dos sarcômeros e do sarcolema (Sandri e Carraro, 1999), porém cada qual com características próprias (Figura 2).

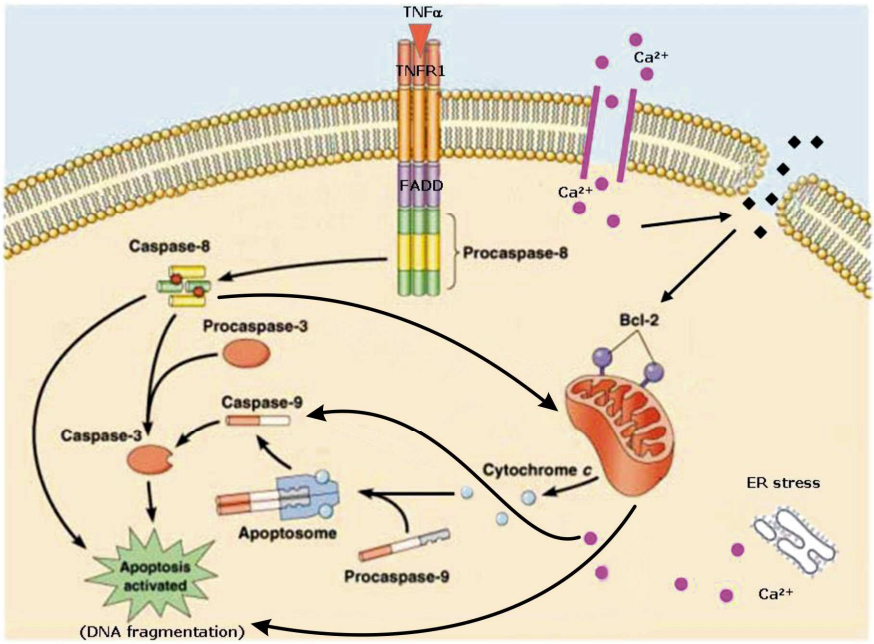

Figura 2: Esquema indicando as principais vias da apoptose. (Adaptado de www.sigmaaldrich.com/img/assets/6460/mit_apoptosis.gif)

Alterações estruturais

Os sarcômeros são as estruturas mais afetadas por substâncias miotóxicas (Gutierrez et al, 1990). Dentre as proteínas estruturais a titina é uma das primeiras a ser destruída quando o músculo é exposto a miotoxinas ou enzimas proteolíticas (Bullard et al, 1990). Entretanto, não se sabe se os processos degenerativos iniciam-se pela desorganização dessa proteína ou se a degeneração é iniciada pelo rompimento do citoesqueleto ou do sarcolema. A titina é uma das proteínas estruturais mais importantes no músculo, garantindo elasticidade e estabilidade à fibra muscular, além de formar uma ancoragem com múltiplos sítios para proteínas sinalizadoras (Liversage et al, 2001; Peng et al, 2005), tendo a função de manutenção da estriação da fibra muscular esquelética e cardíaca (Peng et al, 2005) (Figura 3).

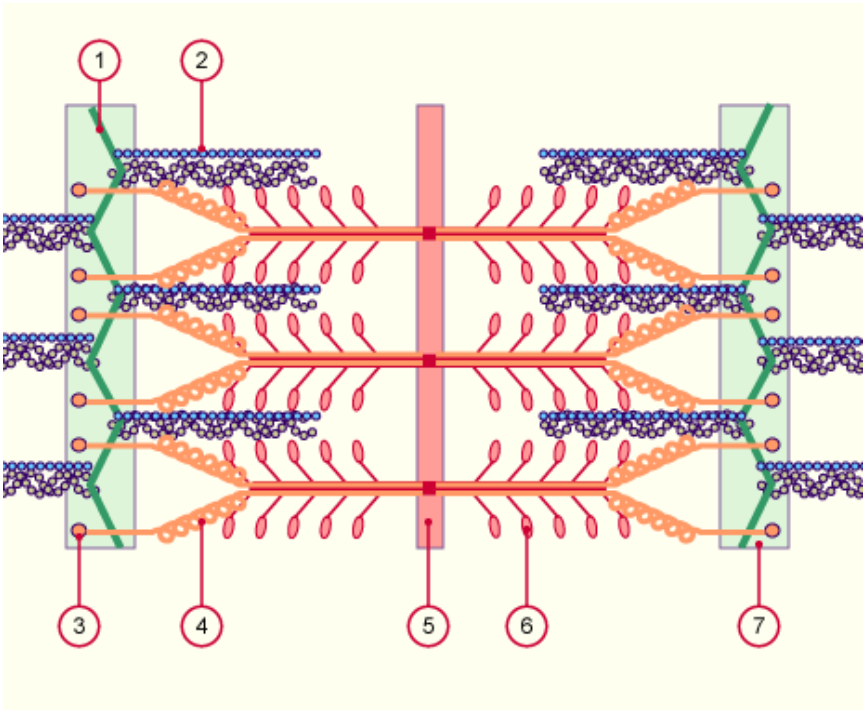

Figura 3: Esquema indicando as principais proteínas estruturais constituintes do sarcômero. 1. α-actinina, 2. nebulina, 3. teletonina, **4. titina**, 5. linha M, 6. cabeça de miosina, 7. linha Z. (www.unifr.ch/.../muskel/skelett/d-skelett.php)

A distrofina está localizada na região subsarcolemal (Bonilla et al, 1988) e, associada a outras proteínas, forma o complexo citoesqueleto-matriz-transmembrana, denominado complexo distrofina-glicoproteína. Embora muitos estudos tenham revelado a estrutura e as interações da distrofina, pouco se sabe sobre o mecanismo no qual sua ausência leva à mionecrose. Sabe-se que sua ausência diminui a estabilidade do sarcolema, aumentando a concentração de Ca^{2+} e levando a ativação de proteases dependentes de Ca^{2+}, como a calpaina, sugerindo que a distrofina pode atuar na estabilização do sarcolema e na regulação da atividade dos canais de Ca^{2+} (Bodensteiner e Engel, 1978; Jackson et al, 1985; Lansman e Franco, 1991; Menke e Jockusch, 1991; Marques, 2004) (Figura 4).

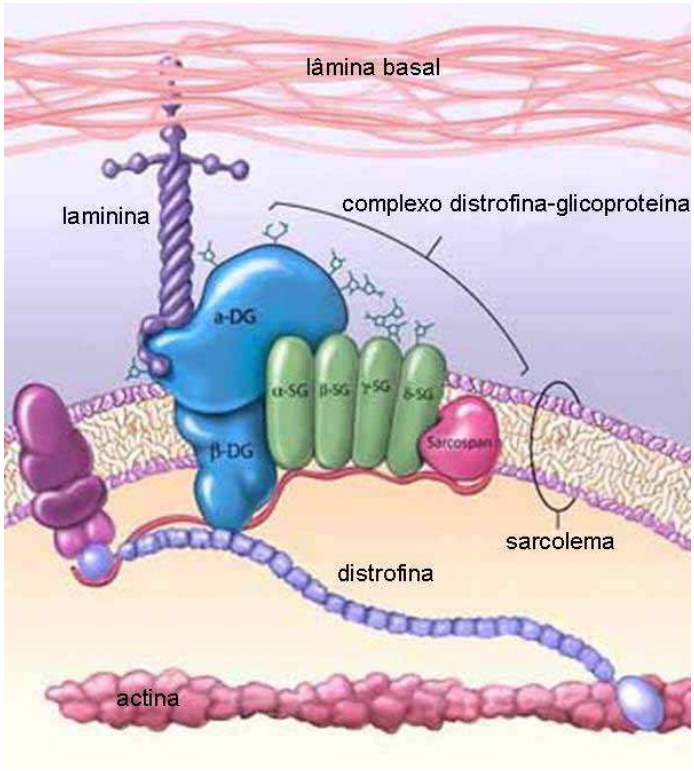

Figura 4: Esquema indicando o complexo distrofina-glicoproteína: lâmina basal, laminina, sarcolema, **distrofina** e actina.
(www.sfn.org/SiteObjects/published/0000BDF20016F63800FD712C3158BA55/0000BDF200000625010EBEBCAE379706/file/bb_image_large.jpg)

Junção Neuromuscular

Como citado anteriormente, acidentes com Hymenoptera, incluindo vespas, podem ocasionar reações graves como broncoespasmo, hipotensão, colapso cardiovascular, perda de consciência (Hamilton et al, 2001; Schmidt, 1995; Tasic, 2000), choque anafilático (Steen et al, 2005), e raros casos de isquemia cardíaca e encefalomielite (Boz et al, 2003), sugerindo que componentes do veneno destes insetos possuem ação no sistema nervoso central (SNC) e periférico (SNP).

Alterações nas concentrações iônicas e permeabilidade seletiva mediada pelos canais iônicos resultam na diferença de potencial elétrico entre o citoplasma e o meio externo e em um gradiente eletroquímico através da membrana plasmática para cada espécie iônica (Hille, 1992). Estes gradientes eletroquímicos desempenham várias funções

celulares, incluindo a geração e propagação de potenciais de ação. Durante a contração do músculo estriado esquelético, controlado pelo SNP através da inervação motora, um potencial de ação gerado na área motora é conduzido por motoneurônio eferentes até a região efetora, o terminal pré-sináptico. Os canais de cálcio situados na porção terminal, dependentes de voltagem, são ativados e promovem o influxo de íons Ca^{2+} para o interior do terminal nervoso (Goldin et al, 2000).

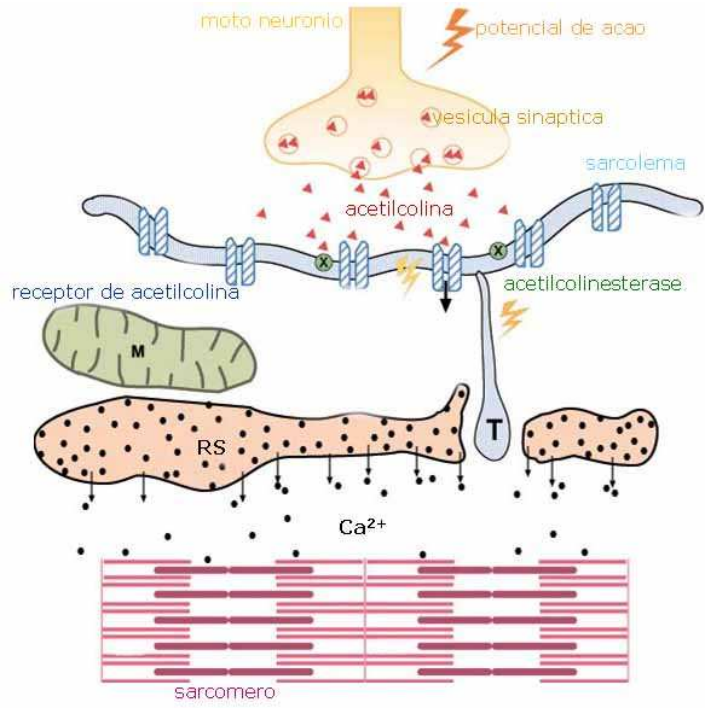

Figura 5: Esquema da junção neuromuscular (JNM): ativação da contração muscular pela excitação do SNP.

O aumento da concentração intraterminal de cálcio desencadeará uma série de mecanismos que resultarão na exocitose de vesículas contendo o mediador químico acetilcolina (ACh) na fenda sináptica. A acetilcolina poderá se difundir para áreas adjacentes sendo degradada pela acetilcolinesterase ou se ligar a receptores específicos (receptores nicotínicos) presentes na membrana pós-sináptica (Dale et al, 1968). A ligação do neurotransmissor ao receptor promove aumento da condutância do canal, o que permitirá o influxo de íons sódio a favor do seu gradiente eletroquímico. Efluxo de íons potássio e influxo de íons Na^{+} resultam na despolarização da membrana pós-sináptica

(Hille, 1992) e propagando uma onda de despolarização do axolema extrajuncional, a qual caminhará pelo sistema T e levará à saída de cálcio dos estoques do retículo sarcoplasmático e o acoplamento do complexo acto-miosina (Figura 5).

Os compostos do veneno de Hymenoptera, como os mastoparanos, podem atuar na Ca^{2+}ATPase do retículo sarcoplasmático (RS) e na ativação dos receptores rianodínicos (RyR) (Figura 6) no músculo esquelético, alterando a captação e liberação de Ca^{2+} do RS, respectivamente, durante a contração e o relaxamento muscular (Hirata et al, 2000).

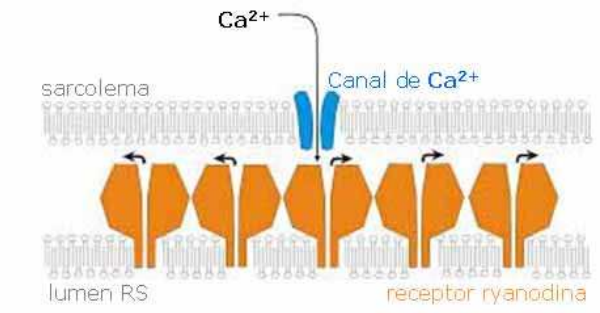

Figura 6: Ryanodina e seus receptores na cisterna terminal do retículo sarcoplasmático. (http://www.bristol.ac.uk/phys-pharm/staffResearch/rebeccasitsapesan/rspic2.jpg)

Justificativa

Sendo a vespa *P. paulista* considerada uma das espécies mais agressivas do estado de São Paulo, e responsável por grande parte dos acidentes com vespídeos, o estudo de componentes do veneno, como o isolamento e a caracterização bioquímica e biológica do mastoparano Polybia-MPII, contribuirá para o entendimento dos mecanismos farmacológicos nos casos de envenenamento. A determinação dos alvos biológicos dessas toxinas pode abrir várias frentes de investigação que terão interesse não só para a pesquisa básica como para a aplicada, com repercussões possíveis na produção de novos fármacos. Os resultados obtidos nesta tese serão parâmetros para pesquisas futuras, uma vez que são poucas as pesquisas realizadas com venenos de Hymenoptera, especificamente mastoparanos isolados de venenos de vespas.

Objetivos Gerais

Avançar no conhecimento da ação do mastoparano Polybia-MPII do veneno de *Polybia paulista* no músculo esquelético e na junção neuromuscular, bem como conhecer aspectos da interação do mastoparano Polybia-MPII com membranas artificiais.

Objetivos Específicos

Investigar a possível ação miotóxica e neurotóxica do mastoparano Polybia-MPII.

Investigar se o mastoparano induz apoptose.

Analisar a ação do mastoparano sôbre lipossomas e seu comportamento em soluções lipossolúveis.

Passos para atingir os objetivos:

Após administração intramuscular do mastoparano Polybia-MPII isolado do veneno de *P. paulista*, no músculo tibial anterior de camundongos Balb/c, pretendeu-se:

- Caracterizar as alterações induzidas no tecido muscular, compreendendo a fase degenerativa (3 e 24 horas – estágios inicial e intermediário) e a regenerativa (3, 7 e 21 dias – estágio tardio), por meio da análise morfológica em microscopia óptica e microscopia eletrônica de transmissão (MET).
- Identificar possíveis eventos de apoptose utilizando-se o método de TUNEL (fragmentação do DNA) e imunohistoquímica e Western Blotting para detecção de caspase 3 e caspase 9.
- Determinar os níveis séricos da enzima Creatino Quinase (CK), um biomarcador padrão de dano da fibra muscular.
- Estudar as alterações induzidas nas proteínas estruturais da fibra muscular, bem como na junção neuromuscular (exocitose de vesículas sinápticas), por meio de imunohistoquímica e MET.
- Estudar os aspectos biofísicos do peptídeo quando de sua interação com soluções lipossolúveis (SDS) e vesículas lipídicas (lipossomos).

(Vide experimentos complementares a partir da página 121)

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Capítulo 1

Myotoxic effects of mastoparan from *Polybia paulista*

(Hymenoptera, Epiponini) wasp venom in mice skeletal muscle

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Myotoxic effects of mastoparan from *Polybia paulista* (Hymenoptera, Epiponini) wasp venom in mice skeletal muscle

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Abstract

In a previous study, we showed that the *Polybia paulista* wasp venom causes strong myonecrosis. This study was undertaken to characterize the myotoxic potency of mastoparan (Polybia-MPII) isolated from venom (0.25 μg/μl) and injected in the tibial anterior (TA) muscle (i.m.) of Balb/c mice. The time course of the changes was followed at muscle degenerative (3 and 24 h) and regenerative (3, 7, and 21 days) periods ($n = 6$) after injection and compared to matched controls by calculation of the percentage of cross-sectional area affected and determination of creatine kinase (CK) activity ($n = 10$). The results showed that although MP was strongly myotoxic, its capacity for regeneration was maintained high. Since the extent of tissue damage was not correlated with the CK serum levels, which remained very low, we raised the hypothesis that the enzyme underwent denaturation by the peptide. Evidence suggested that MP induced the death of TA fibers by necrosis and apoptosis and had the sarcolemma as its primordial target. Given its amphiphilic polycationic nature and based on the vast spectrum of functions attributed to the peptide, we suggest that MP interaction with cell membrane impaired the phosphorylation of dystrophin essential for sarcolemma mechanical stability, and disturbed Ca^{2+} mobilization with obvious implications on sarcoplasmic reticulum and mitochondrial functioning.

Keywords: CK; Muscle fiber vacuolation; Myonecrosis; Sarcolemma disruption; Wasp peptide

1. Introduction

Hymenoptera venoms have been reported to be responsible for 9.3–28.5% of sensitization cases worldwide (Antonicelli et al., 2002). Their stings produce severe pain and local inflammation and represent risk factors for sensitization, local and systemic alterations, and occasionally death in allergic patients (Antonicelli et al., 2002; Steen et al., 2005).

Vespinae venoms contain many different protein components that are biochemically and pharmacologically active, such as phospholipases, hyaluronidases, acid phosphatases, proteases, nucleases (Nakajima et al., 1986; Nicolas et al., 1997; Oliveira and Palma, 1998), and peptides (Nakajima et al., 1986; Konno et al., 2001), the majority of which remains still poorly known in structure.

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The neotropical social wasp *Polybia paulista* (popularly known as "*Paulistinha*") is a very aggressive endemic species in southeastern Brazil, where it frequently causes stinging accidents. Research on the composition and structural characterization of its venom dates back to less than 10 years (Oliveira and Palma, 1998).

Studies in our laboratory indicated that *P. paulista* wasp venom induces blockade of neuromuscular transmission in mouse phrenic nerve–hemidiaphragm preparations. The venom acts on nicotinic receptors, an effect that was partially antagonized by neostigmine, indicating that at least part of their toxins are post-synaptic-acting neurotoxins (Paes-Oliveira et al., 1998). The venom reduces dose dependently and irreversibly the resting potential and the frequency and amplitude of the miniature end-plate potential until complete disappearance of signals, thus indicating the presence of pre-synaptic-acting toxins in the venom (Paes-Oliveira et al., 2000). Light and transmission electron microscopic studies of diaphragm preparations and measurements of creatine kinase (CK) activity in *extensor digitorum longus* muscle preparation indicated that, apart from its post- and presynaptic actions, the *P. paulista* wasp venom exhibits a strong myotoxic effect. The components responsible for these effects have not been identified so far.

Recently, De Souza et al. (2004) identified two toxic peptides in *P. paulista* venom. The primary sequences and the bioassay results suggested that these toxins are members of a new sub-class of mastoparan (MP) peptides directly involved in inflammatory processes. Despite both peptides presenting very potent hemolytic activity and chemotaxis of polymorphonucleated leukocytes, nothing is known about their cellular targets in skeletal muscle and/or the pathogenesis of the local alterations elicited after the MP injection, mimicking wasp sting.

MP, a 14-amino acid amphipathic tetradecapeptide found in venom from different species of wasps, has been known to possess a variety of biological actions, including antimicrobial activity (Mendes et al., 2004), mast cell degranulation (Hirai et al., 1979; De Souza et al., 2004), activation of G-protein-mediated mechanisms (Higashijima and Ross, 1991), stimulation of phospholipases A_2 and C (Perianin and Snyderman, 1989), mobilization of Ca^{2+} from sarcoplasmic reticulum (SR), activation of the ryanodine receptor, modulation of various enzymes, for example, the Na^+-K^+-ATPase of rat brain (Hirata et al., 2000, 2003), induction of the mitochondrial permeability transition (Pfeiffer et al., 1995), and cell death by necrosis and apoptosis (Perianin and Snyderman, 1989).

Skeletal muscle is a subcutaneous tissue that corresponds to approximately 80% of the total mammal body mass (Harris and Cullen, 1990), and so a predictable target in victims of multiple stings induced by Hymenoptera. The current study was undertaken to investigate to what extent the Polybia-MPII mastoparan (INWLKLGKMVIDAL-NH_2) from *P. paulista* venom contributes to the myotoxic action already known of the whole venom in muscle tissue (Paes-Oliveira et al., 1998, 2000). The time course of the muscle changes was followed from 3 h to 21 days to assess the myotoxic potency of MP and pathogenesis throughout the degenerative and regenerative post-envenoming stages using morphological, morphometrical, and biochemical approaches. (Part of this work has been presented at the IX Congress of the Brazilian Society of Toxinology, Fortaleza, CE, 2006.)

2. Material and methods

2.1. Animals

Adult male Balb/c mice (~25 g) were obtained from an established colony maintained by the Animal Services Unit of the State University of Campinas (UNICAMP) and maintained in a temperature-controlled room (20±3 °C) on a 12 h light/dark cycle, with lights on at 6 a.m., and fed standard Purina chow with free access to water.

The experimental protocol was approved by the university's Committee for Ethics in Animal Experimentation (CEEA/UNICAMP) and followed the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985).

2.2. Venom toxin– Polybia-MPII mastoparan (INWLKLGKMVIDAL-NH_2) synthesis

As described by De Souza et al. (2004), two novel inflammatory polypeptides from *P. paulista* venom were characterized. One of them Polybia-MP-II (INWLKLGKMVIDAL-NH_2) was chosen for use in this study. Because a large number of wasp glands are necessary to extract venom and purify MP, we set an analytical protocol of synthesis in

order to optimize the attainment of a sufficient amount of MP.

The peptide was prepared by stepwise manual solid phase synthesis using *N*-9-fluorophenyl-methoxy-carbonyl (Fmoc) chemistry with Novasyn TGS resin (Nova Biochem). Side-chain protective groups included *t*-butyl for serine and *t*-butoxycarbonyl for lysine. Cleavages of the peptide–resin complexes were performed by treatment with trifluoroacetic acid/1,2-ethanedithiol/anisole/phenol/water (82.5:2.5:5:5:5 by volume), using 10 ml/g of complex at room temperature (RT) during 2 h. After filtering to remove the resin, ethyl ether at 4 °C was added to the soluble material causing precipitation of the crude peptide, which was collected as a pellet after centrifugation at 1000*g*, during 15 min at RT.

The crude peptide was suspended in water and chromatographed under reversed-phase HPLC (RP-HPLC) using a semi-preparative column (Shiseido C18, 250 × 10 mm, 5 mm), under isocratic elution with 45% (v/v) acetonitrile in water [containing 0.1% (v/v) trifluoroacetic (TFA)] at a flow rate of 2 ml/min. The elution was monitored at 215 nm with a UV-DAD detector (Shimadzu, mod. SPD-M10A) and each fraction eluted was manually collected into glass vials of 15 ml volume (Fig. 1A). Only the large peak assigned with the black arrow in Fig. 1A was pooled, concentrated, and analyzed for homogeneity and correctness.

The homogeneity and correct sequence of the synthetic peptide were evaluated by comparing their retention times in the RP-HPLC under isocratic conditions with 40% (v/v) MeCN [containing 0.1% (v/v) TFA] against the natural peptide; ESI-MS analysis was also used to check the peptide purity [considering as the criterion the presence of a single molecular ion, equivalent to the expected molecular mass (MW) for the amino sequence] (Fig. 1B); and finally the sequence of the synthetic material was confirmed by automated sequencing based on automatic Edman degradation chemistry. The MW of the natural MP (1613 kDa) and the synthetic peptide (1613.5 kDa) matched.

2.3. Experimental procedure

Mice were deeply anesthetized with a 1:1 mixture of ketamine chloride (Dopalen®, Vetbrands, 100 mg/kg of animal) and xylazine chloride (10 mg/kg, Anasedan®, Vetbrands) (2 µl/mg body weight, i.p.). The right tibial anterior (TA) muscle was exposed, injected intramuscularly (i.m.) into the middle third with 0.25 µg/µl of MP from *P. paulista* venom in a volume of 100 µl of physiological saline solution, after which the surgical wound was closed. The toxin concentration was chosen based on the estimated concentration of this peptide in an amount of venom corresponding to the volume injected during 1–3 wasp stings. Control animals were injected with saline solution only (sham group).

After 3 and 24 h, 3, 7, and 21 days, mice from the saline and MP groups were again anesthetized, and used both for blood sampling destined to CK measurements (see Section 2.5) and for TA muscle dissection for morphological and quantitative studies (see Section 2.4). At each time point after muscle dissection and blood collection, the anesthetized mice were killed by cervical dislocation.

2.4. Histopathological analysis and quantification of myonecrotic and regenerative area

The excised muscles were maintained in 4% paraformaldehyde fixative overnight, washed three times in 0.05 M PBS, dehydrated in increasing concentrations of ethanol (70%, 90%, and 100%), and embedded in paraffin. Sections of 5 µm thickness were cut using a Leica RM 2035 microtome and stained with toluidine blue (TB) and Mallory's trichrome (MT) for examination by light microscopy. All analyses were done with an Olympus BX51 light microscope. The percentage of muscle damage was assessed by multiplying the necrotic area by 100 and dividing it by the whole TA cross-sectional area in saline- and MP-injected mice at each time point scheduled ($n = 6$ mice/period). Similarly, the percentage of regenerating area along periods of 3, 7, and 21 days was assessed by multiplying the regenerative area by 100 and dividing it by the whole cross-sectional area of the muscle. Evaluation of the unaltered area of the muscle was shown by simply subtracting the damaged and/or regenerated area from the whole section of the muscle. From all experimental groups, three cross sections were always taken just in the vicinity of the site of the injection of MP or saline solution. Sections that had their areas measured were 50 µm distant from each other, although the determination of the area was done in 15 sections per time interval collected in a central strip of tissue (150 µm width) of the muscle belly.

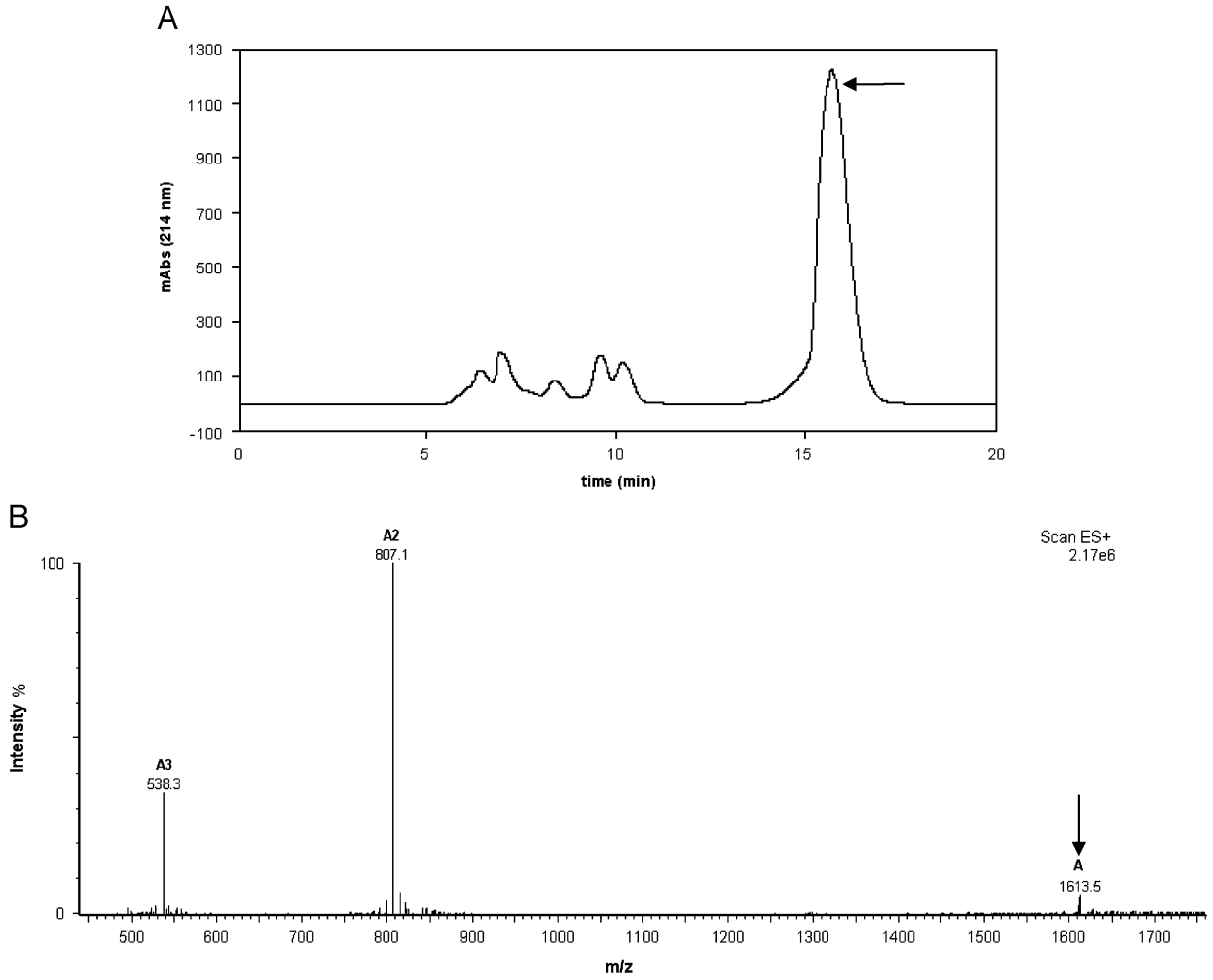

Fig. 1. (A) RP-HPLC chromatogram of *P. paulista* synthetic mastoparan Polybia-MP-II (INWLKLGKMVIDAL-NH_2) obtained with a C-18 column (10 × 250 mm), under isocratic elution with 45% MeCN (v/v) containing 0.1% TFA (v/v). The arrow represents the purified peptide. The retention time of the peptide was 15.7 min. (B) ESI mass spectrum for the synthetic mastoparan (INWLKLGKMVIDAL-NH_2) obtained by ESI/MS analysis. The [M + H] +, [M + 2H] 2+, and [M + 3 H] 3+ ions were labeled as A, A2, and A3, respectively. The arrow represents the purified peptide in the monoprotonated form.

2.5. Serum creatine kinase (CK) activity

Supplementary quantification of myonecrosis was assayed by measuring the CK serum activity in both saline- and MP-injected mice ($n = 10$ mice/period). Three and 24 h, 3, 7, and 21 days after injection, the mice were deeply anesthetized and blood samples were collected by cardiac and/or infraorbital plexus puncture into heparinized capillary tubes. Immediately after blood sampling, it was centrifuged (4 °C, 3000 rpm/10 min) for serum separation and colorimetric determination of CK activity (CK-NAC kit, Bioclin; CK-NAC kit, Wiener Lab.). The serum CK activity was expressed as international units/liter (U/L), with 1 unit of activity causing the phosphorylation of 1 μmol of creatine (substrate) per minute, at 25 °C. From the 10 mice used for CK assays, 6 were used for morphology (Section 2.4).

2.6. Statistical analysis

All data were presented as means ± SEM of the number of animals used or experiments done. Statistical inter-group comparisons of the relative myonecrotic area and serum CK levels were done using one-way analysis of variance (ANOVA) and the Tukey–Kramer multiple comparisons test, with $P < 0.05$ indicating significance.

3. Results

3.1. Histopathological analysis and morphometry

The criteria used to consider an area as damaged was the presence of hemorrhage, inflammatory infiltrate, and degenerating muscle fibers. Damaging area was measured only at 3 and 21 h because thereafter regenerating fibers appeared intermingled among the groups of necrotic fibers. The criterion for considering an area as regenerating was basically the presence of central nuclei fibers, independent of its profile size and independent of the presence of advanced necrotic cells interspersed among the regenerating ones. Regenerating area was measured at 3, 7, and 21 days.

The histopathological changes were qualitatively and quantitatively different between control and envenomed experimental groups and among some envenomed groups themselves in the different time points analyzed (Fig. 2). Significant differences between the mean cross-sectional area comprised of damaged tissue in saline- and toxin-injected mice at 3 and 24 h were seen: $0.5 \pm 0.6\%$ vs $76.5 \pm 11.4\%$ ($P < 0.01$), and $0.6 \pm 0.6\%$ vs $50 \pm 19.5\%$ ($P < 0.05$), respectively (Fig. 2A). Differences between saline- and MP-injected TA regenerative area were also observed in all time points (3–21 days) (Fig. 2B). Whereas saline controls presented the great majority of fibers with normal morphology (Fig. 3A), MP-injected TA presented fibers with different pathologic states, including vacuolated (Fig. 3B), swollen (Fig. 3C), and heavily stained (Fig. 3D) fibers, with delta lesions (Fig. 3C), with loosely clumped or densely clumped myofibrils (Fig. 3C and D), and "ghost" fibers (Fig. 3D). The majority of these alterations involved sarcolemma rupture. However, many necrotic fibers characterized by the presence of multiple vacuoles did not show any membrane disruption (see Fig. 3B). In addition, whereas in control saline-injected TA well-organized fascicles with fibers disposed close together were observed, in MP-injected TA the fascicles presented dispersed fibers, among which inflammatory

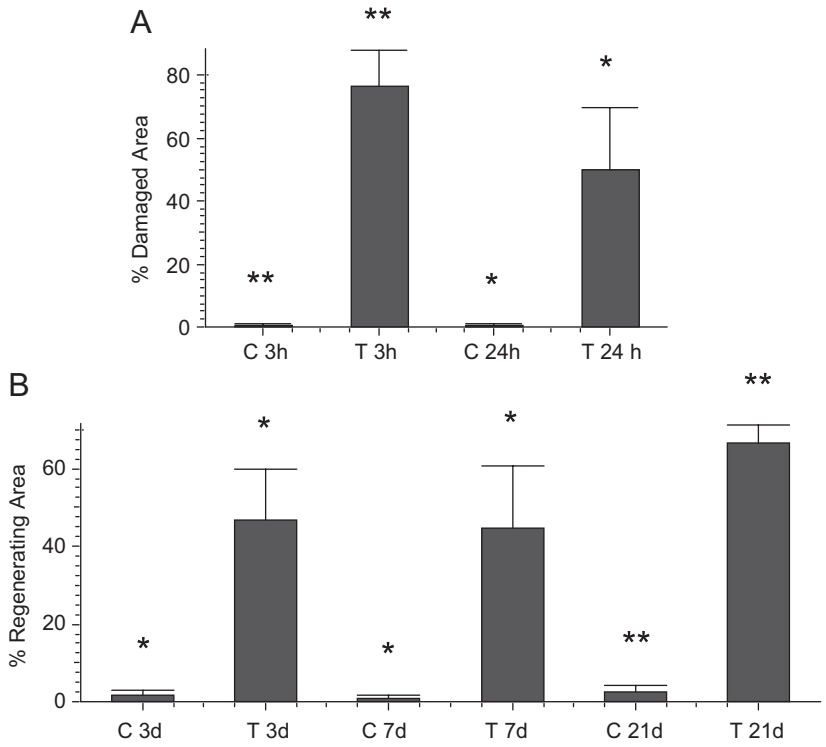

Fig. 2. The results showed significant differences (A) between the mean cross-sectional area comprised of damaged tissue in saline- (C) and mastoparan-injected (T) muscles at 3 and 24 h. (B) Comparison of regenerative fiber-containing area in control (C) and envenomed (T) groups showed a significant gradual increase in their number along the time intervals studied (3, 7, and 21 days). One-way ANOVA, $^{*}P < 0.05$; $^{**}P < 0.01$.

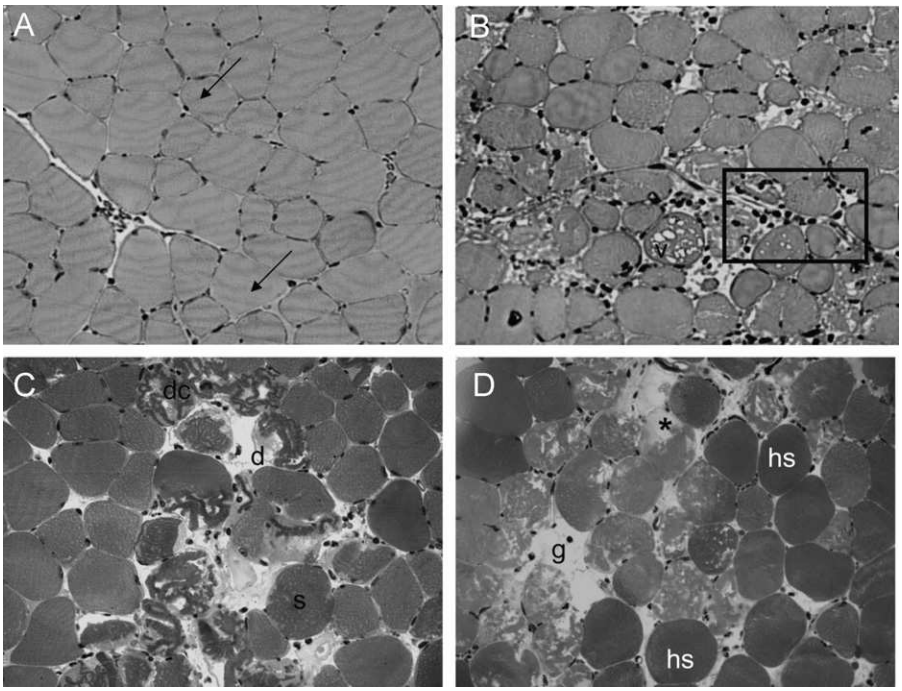

Fig. 3. Light micrographs of sections of TA muscle taken after injection of 0.25 μg/μl (i.m.). (A) Transverse section showing the general aspect of control muscles. Note the polygonal fibers aspect (arrows) and the nucleus in the fiber periphery. (B–D) The damaged area was seen after 3 and 24 h of envenoming. Note the vacuolated (v), swollen (s), and heavily stained (hs) fibers, with delta lesions (d), loosely clumped or densely clumped myofibrils (–*), "ghost" fibers (g), and inflammatory infiltrating cells (square).

infiltrating cells and extravascular erythrocytes were found (Fig. 3B).

The calculation of the cross-sectional regenerating area revealed that it passed gradually from 46.6± 13.4% to 44.6±16.2% to 66.5±4.7% at 3, 7, and 21 days, respectively (Fig. 2B). Figs. 4A–F illustrate the TA morphology from 3 to 21 days, indicating that regeneration was underway. Comparison between control and envenomed groups showed a significant increase of regenerating fibers in all time points (Fig. 2). An interesting point to mention is that a few sponge-like necrotic fibers not seen before appeared at day 7 after envenoming (Fig. 4D).

3.2. Serum creatine kinase (CK) activity

The original serum CK values (x_i) were heteroskedastic, i.e., the variances within groups were heterogeneous. Therefore, it was not possible to use the ANOVA. In order to analyze these values statistically, it was necessary to make the variances homoskedastic, using the equation

$$\log(x_i) = \log(\mathrm{CK}) = y_i,$$
$$y_i = \ln(x_i).$$

This equation attended to the requirement for the ANOVA test, in which variance within groups has to be homogeneous (homoskedastic) (Table 1). The oscillation of the serum CK values of control and MP-treated animals at each time point presented similar profiles. In controls, the serum CK activity oscillated from 5.71 to 3.84 log(CK) U/L, whereas in envenomed animals it oscillated from 6.01 to 4.60 log(CK) U/L from 3 h to 21 days. However, no significant differences were observed ($P > 0.05$) between control and envenomed groups (Fig. 5).

4. Discussion

The results presented in this study indicate that MP from *P. paulista* wasp venom injected in the TA muscle (i.m.), mimicking the sting inflicted in victims, induces myonecrosis at the site of injection. At acute time periods (3 h) after i.m. toxin injection, myofibers exhibited a range of pathologic states, whose pattern was also seen at 24 h. However, the extent of damage decreased about a quarter at 24 h, a fact that was attributed to edema and hemorrhage regression.

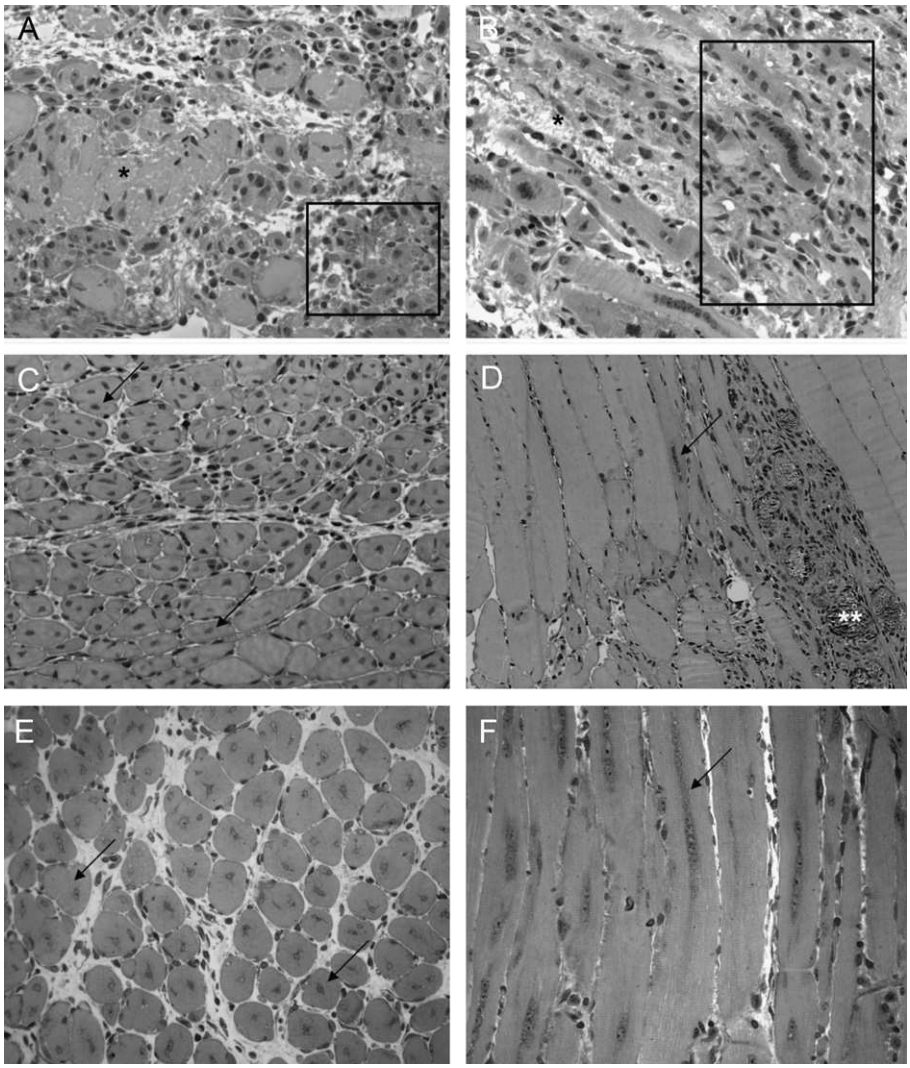

Fig. 4. General aspects of TA muscle after injection of 0.25 μg/μl (i.m.) mastoparan—regenerative phase. (A, B) 3 days: Note the small regenerating cells with central nucleus (square). Some damaged fibers (*) still persisted. (C–F) 7 days: Note the greater size of regenerative fibers after 7 days (arrow). A few sponge-like necrotic fibers not seen before appeared at day 7 after envenoming (**). (E, F) 21 days: At 21 days, the regenerated fibers were bigger than those at 7 days, but the nucleus continued to be central (arrow).

Table 1
CK values and corresponding log(CK) values

Time	Saline 0.9%		Mastoparan 0.25 μg/μl	
	CK	log(CK)	CK	log(CK)
3 h	302±1.65	5.7100±0.50	410±1.66	6.0170±0.51
24 h	175±2.01	5.1645±0.70	259±2.03	5.5567±0.71
3 d	94±1.43	4.5445±0.36	169±2.11	5.1301±0.75
7 d	121±2.24	4.7969±0.81	220±2.46	5.3933±0.90
21 d	47±2.03	3.8493±0.71	100±1.50	4.6084±0.40

Data were expressed by mean±SD in U/L. Statistical significance was set at $P \leqslant 0$. No difference in CK values was found.

The kind of pathological states presented by the fibers were also observed in diaphragm muscle preparations incubated with the whole venom of the species (Paes-Oliveira et al., 1998, 2000). Studies in our laboratory showed that phospholipase isolated from the venom of *P. paulista* also has myotoxic activity, however much less potent than that exhibited by MP (unpublished results), suggesting that MP is the major myotoxic component of the venom.

The majority of necrotic lesions elicited by MP in mice TA muscle studied here seemed to be

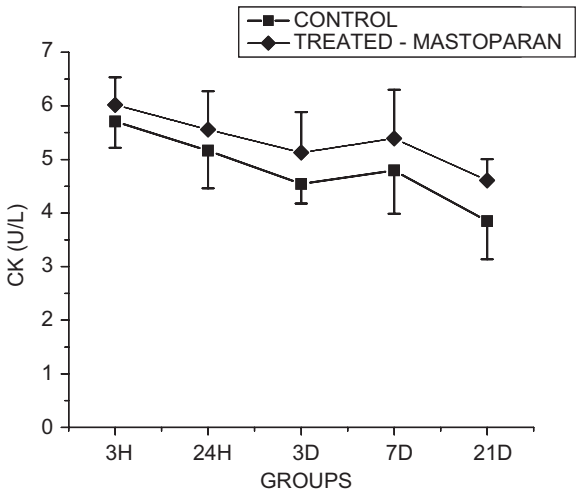

Fig. 5. CK levels in control- and mastoparan-injected (0.25 μg/μl) TA muscle. The oscillation of the serum CK values between both groups at each time point presented similar profiles. However, no significant difference was observed ($P>0.05$) between control and envenomed groups.

associated with the rupture of the sarcolemma followed by rapid disorganization of the myofibrils. To a lesser extent, necrotic fibers without sarcolemma disruption, but multi-vacuolated, were also present. The mechanism underlying the myonecrotic process is unknown, and was not investigated in the present study. Nonetheless, some discussion was addressed to putative mechanisms involved in the generation of the structural alterations seen.

MP has been described as a bioactive peptide found in wasp venom, which interacts with cell plasma membrane and induces changes in its biological properties. Like other basic naturally occurring amphiphilic peptides, such as mellitin (from bee venom), cardiotoxin (from cobra venom), and polymyx B (antibacterial antibiotic), as well as the ether lipid ET-18-OCH_3, MP inhibits protein kinase C (PCK) under different activation conditions (Raynor et al., 1991). Dystrophin, a component of the membrane cytoskeleton of striated muscle, bound to actin myofilaments is known to be phosphorylated by endogenous protein kinases, among which PCK is included. The phosphorylation of dystrophin was activated by cAMP, cGMP, calcium, and calmodulin, and was inhibited by cAMP-dependent protein kinase peptide inhibitor, among which MP and heparin are included (Raynor et al., 1991; Luise et al., 1993). A large amount of evidence points to the importance of phosphorylation as a translational event implicated in modulation of protein–protein interactions and stabilization of the fiber cytoskeleton (Senter et al., 1995). Here we suggest that the impairment of dystrophin phosphorylation deteriorates the role of dystrophin as a linker between the muscle fiber cytoskeleton and sarcolemma. As a result, the necessary mechanical stability of the membrane is lost, leading to rupture of the membrane. To ensure that these mechanisms in fact underlie the myotoxic effects of MP, further studies will be necessary.

As mentioned before, a number of necrotic fibers observed after 3 and 24 h did not show sarcolemma disruption but numerous vacuoles inside. Vacuolated fibers induced by MP are identical to that caused by cardiotoxins found in elapid venom. Cardiotoxins were described to induce leakage in the muscle sarcolemma (Duchen et al., 1974) and allow influx of Na^+ and water (Ownby et al., 1976), which is then captured by SR and render the fibers vacuolated. It has been shown that MP inhibits Na^+-K^+-ATPase, probably by directly or indirectly interacting with Na^+-binding sites located primarily at the cytoplasmic site of plasma membranes, and with Ca^{2+}/calmodulin-dependent protein kinase II (Raynor et al., 1992). These findings were consistent with discrete interactions of MP with functionally distinct sites on the membrane, leading to differential inhibition of membrane biological activities (Hirata et al., 2000, 2003). These authors reported that MP activates the ryanodine receptor involved in Ca^{2+} release from skeletal muscle SR, known to play a central role in the excitation–contraction coupling of skeletal muscle. The authors showed that the activation of ryanodine receptors involves a 97-kDa MP-binding protein present in the receptors of the SR heavy fraction isolated from rabbit skeletal muscle (Hirata et al., 2000). On the other hand, it was shown that MP is a potent inhibitor of the SR Ca^{2+}-ATPase (SERCA), an enzyme serving an important function during muscle relaxation by transporting intracellular calcium into the lumen of the SR coupled to ATP hydrolysis. Inhibition of SERCA was reported to be induced by myotoxin *a*, from rattlesnake venom, and melittin, isolated from bee venom, both, like MP, cationic peptides (Longland et al., 1999). MP decreases the affinity of the ATPase for Ca^{2+} and abolishes the cooperation of Ca^{2+} binding. Calcium ion release from SR induced by MP is not caused by the inhibition of Ca^{2+} pump (Longland et al., 1998, 1999). We suggest that disturbances on sodium and calcium mobilization likely could also

be behind the effects seen in this study in mice TA injected with MP.

Myonecrosis has been classically measured by determination of CK activity. Concomitant histological analysis associated with morphometry has allowed assessment of muscle structural damage. CK (E.C.2.7.3.2), a critical enzyme present in high-requirement cell types, such as skeletal muscle (Wallimann et al., 1992), catalyzes the reversible transphosphorylation reaction ATP + creatine ↔ ADP + phosphocreatine. The enzyme, which is found in cytosolic and mitochondrial isoforms (Wyss et al., 1992), is involved in transport of energy from the mitochondrion and within the cytoplasm. Structurally, the cytosolic isoform is associated to myofibrils M-line and SR membranes, whereas functionally it is coupled to ATPase, and thus in a strategic position to take part in the functioning of the contractile muscle fiber apparatus and SR Ca^{2+} mobilization (Wallimann and Eppenberger, 1985).

In the present study, the CK values measured at 3 and 24 h did not reflect the significant difference found in the percentage of the damaged cross-sectional area seen in control and MP-injected muscles, and between envenomed groups themselves ($P<0.001$). In the same way, no significant difference was seen in CK serum activities at 3, 7, and 21 days, despite the significant difference obtained with the measurement of the regenerating tissue area ($P<0.001$). An explanation for these conflicting results is unknown. However, a possibility that the enzyme could have its active sites inactivated by MP is raised. Denaturation of CK seems to occur by change in the conformation of the active catalytic site, shown to be more easily perturbed than the molecule as a whole (Zhou et al., 1993). On the other hand, some studies have shown that serum levels of cytosolic proteins, such as CK and myoglobin, do not necessarily reflect the amount of structural damage of muscle tissue, different from structurally bound proteins such as myosin heavy chain and troponin which better express myofibril breakdown (Sorichter et al., 1999). In our study, a meaningful explanation for the lack of correspondence between muscle-damaged area and CK activity is lacking, although the hypothesis of an MP-inactivating enzyme action is attractive.

Among the broad range of pharmacological activities exerted by MP, another refers to the opening of mitochondrial permeability transition (MPT) large pores in the inner mitochondrial membrane, apparently by bilayer membranes perturbation, increased calcium load, and redox stress (Pfeiffer et al., 1995; Lemasters et al., 1998; Armstrong, 2006). Recent works have shown the link between MPT with cell injury mechanisms culminating with cell death by autophagy, necrosis, and apoptosis (Lemasters et al., 1998; for reviews, see Amacher, 2005; Armstrong, 2006).

The vast literature about MP effects indicates that the peptide affects pivotal cell functions. Considering that sarcolemma, sarcotubular system, and mitochondria, shown to be targets for MP, are leading organelles in skeletal muscle fiber functioning, it is unsurprising to attribute to this peptide the major myotoxic activity exhibited by the whole venom.

In conclusion, our results show that Polybia-MPII mastoparan is a potent myotoxin probably having as principal targets the sarcolemma, including the transverse T system, SR, and mitochondria, possibly by affecting directly or indirectly dystrophin and Ca^{2+} mobilization. The hypothesis is that this effect occurs by inhibition of different kinases, and interference on calcium release from and recapture by SR. Disturbance of the calcium homeostasis can promote mitochondria permeability transition, loss of mitochondrial and SR ion buffering roles, and eventually cell death by necrosis and apoptosis, as seen in this study.

As inferred from the literature, there are many mechanistic pathways possibly involved in MP effects on skeletal muscle. Each of the time points of the pathogenesis here followed probably addresses some of these pathways. The amphiphilic and polycationic nature of the MP enables the molecule to change conformation, to promote a detergent effect on the membrane surface, and to interact with anionic molecules within the cell. The outcome of this interaction was a partial myolytic effect, which preserves a rather faster regeneration of muscle fibers, different from the regeneration seen after i.m. injection of phospholipase of *P. paulista* venom (unpublished results).

Acknowledgments

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Capítulo 2

Mastoparan Effects in Skeletal Muscle Damage: An ultrastructural view until now concealed

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Microscopy Research and Technique 71:220-229 (2008)

Mastoparan Effects in Skeletal Muscle Damage: An Ultrastructural View Until Now Concealed

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KEY WORDS *Polybia paulista*; polybia-MPII peptide; tibial muscle; mitochondria; sarcolemma

ABSTRACT Animal venoms have been valuable sources for development of new drugs and important tools to understand cellular functioning in health and disease. The venom of *Polybia paulista*, a neotropical social wasp belonging to the subfamily Polistinae, has been sampled by headspace solid phase microextraction and analyzed by gas chromatography-mass spectrometry. Recent study has shown that mastoparan, a major basic peptide isolated from the venom, reproduces the myotoxic effect of the whole venom. In this study, Polybia-MPII mastoparan was synthesized and studies using transmission electron microscopy were carried out in mice tibial anterior muscle to identify the subcellular targets of its myotoxic action. The effects were followed at 3 and 24 h, 3, 7, and 21 days after mastoparan (0.25 μg/μL) intramuscular injection. The peptide caused disruption of the sarcolemma and collapse of myofibril arrangement in myofibers. As a consequence, fibers presented heteromorphic amorphous masses of agglutinated myofilaments very often intermingled with denuded sarcoplasmic areas sometimes only surrounded by a persistent basal lamina. To a lesser extent, a number of fibers apparently did not present sarcolemma rupture but instead appeared with multiple small vacuoles. The results showed that sarcolemma, sarcoplasmic reticulum (SR), and mitochondria were the main targets for mastoparan. In addition, a number of fibers showed apoptotic-like nuclei suggesting that the peptide causes death both by necrosis and apoptosis. This study presents a hitherto unexplored view of the effects of mastoparan in skeletal muscle and contributes to discuss how the known pharmacology of the peptide is reflected in the sarcolemma, SR, mitochondria, and nucleus of muscle fibers, apparently its subcellular targets. *Microsc. Res. Tech. 71:220–229, 2008.*

INTRODUCTION

Among the medically important Hymenoptera, wasps deserve special attention because in contrast to bees, which die after stinging, they can sting multiple times. Human accidents can be lethal for their victims. Immediate hypersensitivity to venom components can lead individuals of a single wasp stinging to death by anaphylaxis. Nonallergic patients can also die after massive stinging of these insects (Fitzgerald and Flood, 2006).

The venom of Hymenoptera contains numerous pharmacologically active substances which comprise high molecular weight proteins such as enzymes, allergens, and toxins, amines and small basic peptides (Habermann 1972; Nakajima, 1984). Such substances are able to cause mast cell degranulation (mastoparan), hemolysis (melittin), neurons death (apamin), pain-generation (kinins, e.g., vespakinins, polisteskinins, Thr^6-bradykinin, Ala-Arg-Thr^6-bradykinin), and chemotaxis of polymorphonuclear leukocytes, among others (Murata et al., 2006). Much of these substances share these bioactive effects, for instance mastoparan can cause hemolysis, chemotaxis, histamine degranulation (De Souza et al., 2004), and neurotoxic effects. Because their bioactive effects may act on very specific targets, these substances have long contributed to understand biological processes.

Mastoparans (MP), the major component of wasps' venom, are cationic tetradecapeptides rich in hydrophobic amino acids, whose first role described was to provoke degranulation of mast cells and consequent release of histamine (Hirai et al., 1979). In the late decades, a series of reports have reiterated MP as potent stimulator of exocytosis of secretory granules from various epithelial or connective cells, such as catecholamines from chromaffin cells, prolactin from adenohypophysis cells, serotonin from platelets, and histamine from peritoneal mast cells (Katsu et al., 1990; Kurihara et al., 1986; Seebeck et al., 2001). In pancreatic islets, MP is stimulator of insulin and glucagon exocytosis through GTP-binding regulatory protein(s) coupled with phospholipases A and C activation, and intracellular Ca^{2+}liberation (Yokokawa et al., 1989, Komatsu et al., 1992). In muscle, MP increases p38 mitogen-activated protein kinase activity, cytosolic phospholipase A_2 (PLA_2) activity, and arachidonic acid release from smooth muscle cells

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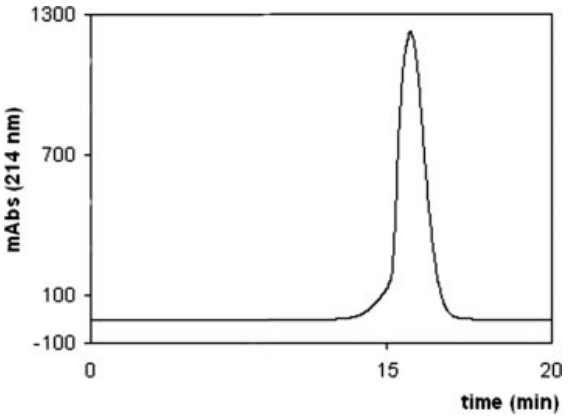

Fig. 1. RP-HPLC chromatogram of *P. paulista* synthetic Polybia-MPII mastoparan (INWLKLGKMVIDAL-NH_2) with a C-18 column (10×250 mm^2), under isocratic elution with 45% (vol/vol) MeCN, containing 0.1% (vol/vol) TFA. Retention time of peptide was 15.7 min.

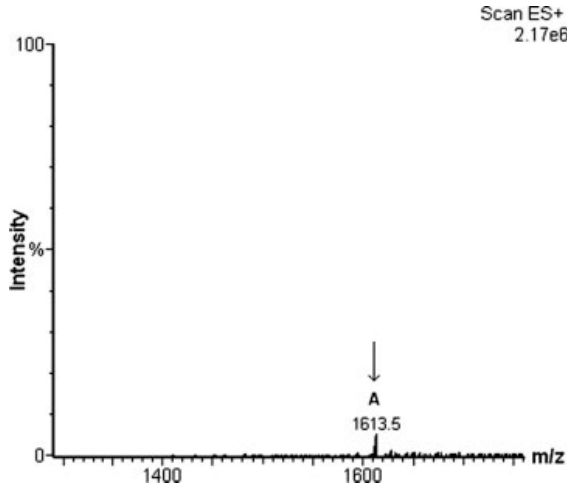

Fig. 2. ESI mass spectra for the synthetic mastoparan (INWLKLGKMVIDAL-NH_2) obtained from the ESI/MS analysis. The [M+H] + ion is labeled as A (arrow). The arrow represents the purified peptide.

(Husain and Abdel-Latif, 1999), and induces atrial natriuretic factor release from atrial cardiomyocytes (Bensimon et al., 2004). In both muscle cells the effects of MP were also shown to be mediated by G proteins.

A broad amount of studies related with Hymenoptera venoms or their components have addressed their focus on the allergenic stinging reactions (Perez-Pimiento et al., 2005), allergen constituents (Hoffman, 2006), biochemical composition (Hisada et al., 2005; Mendes et al., 2004), mechanisms associated with pain generation, and inflammatory reactions (De Paula et al., 2006), just to cite some of them.

Specially, in relation to MP more than 600 studies are found in literature. None of them has dealt with the effects of the peptide in the ultrastructural components of skeletal muscle cells. Studies with the crude venom of *Polybia paulista* on mouse nerve-muscle preparations have shown that venom possesses pre- and postsynaptic acting neurotoxins leading to blockade of the neuromuscular transmission and causes myonecrosis in mice diaphragm (Paes-Oliveira et al., 1998, 2000). *P. paulista* (Hymenoptera; Vespidae) is an aggressive social wasp commonly found in Brazil. It belongs to the subfamily Polistinae, the largest and more diverse group of social wasps, not only in regard to the number of species (distributed in nine genera and four tribes), but also in regard to its morphological and behavioral diversity (Richards, 1978; Carpenter, 1991).

In a recent study, the Polybia-MPII mastoparan (INWLKLGKMVIDAL-NH_2) from *P. paulista* wasp venom (De Souza et al., 2004) was shown to present myotoxic action in mice tibial anterior (TA) muscle (Rocha et al., 2007). In this work, using a similar experimental protocol, an ultrastructural view of the effects of mastoparan in skeletal muscle tissue, until now concealed, was intended to investigate which are the main subcellular targets of the peptide.

MATERIALS AND METHODS

Animals

Adult male Balb/c mice (~25 g) were obtained from an established colony maintained by the Animal Services Unit of the State University of Campinas (UNICAMP) and maintained in a temperature-controlled room ($20 \pm 3^{\circ}C$) on a 12 h light/dark cycle with lights on at 6 A.M. and fed standard Purina chow with free access to water. The experimental protocol was approved by the university's Committee for Ethics in Animal Experimentation (CEEA/UNICAMP) and followed the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985).

Peptide Synthesis

As described by De Souza et al. (2004), two novel inflammatory polypeptides from *P. paulista* venom were characterized. One of them, the Polybia-MPII mastoparan (INWLKLGKMVIDAL-NH_2) presented stronger hemolytic and mast cell degranulation activities and was elected to be used in this study. Because a large number of wasp glands would be necessary for extracting venom and purify the peptide, we had set an analytical protocol of synthesis in order to optimize the attainment of a sufficient amount of Polybia-MPII mastoparan.

The peptide was prepared by step-wise manual solid phase synthesis as described by Rocha et al., (2007). The crude peptide was suspended in water and chromatographed under reversed-phase HPLC (RP-HPLC) using a semipreparative column (Shiseido C18, 250×10 mm^2, 5 μm), under isocratic elution with 45% (vol/vol) acetonitrile in water [containing 0.1% (vol/vol) trifluoroacetic, TFA] at a flow rate of 2 mL/min, during 20 min. The elution was monitored at 215 nm with a UV-DAD detector (Shimadzu, mod. SPD-M10A), and each fraction eluted was manually collected into glass vials of 15 mL volume. Only the largest fraction was pooled concentrated and analyzed for homogeneity (Figure 1).

The correct sequence of the synthetic peptide was evaluated by Automatic Edman Degradation Chemistry and ESI-MS analysis was also used to check the peptide purity (considering as criteria the presence of a single molecular ion, equivalent to the expected molecular mass (MW) for the amino sequence) (Fig. 2). The MW of

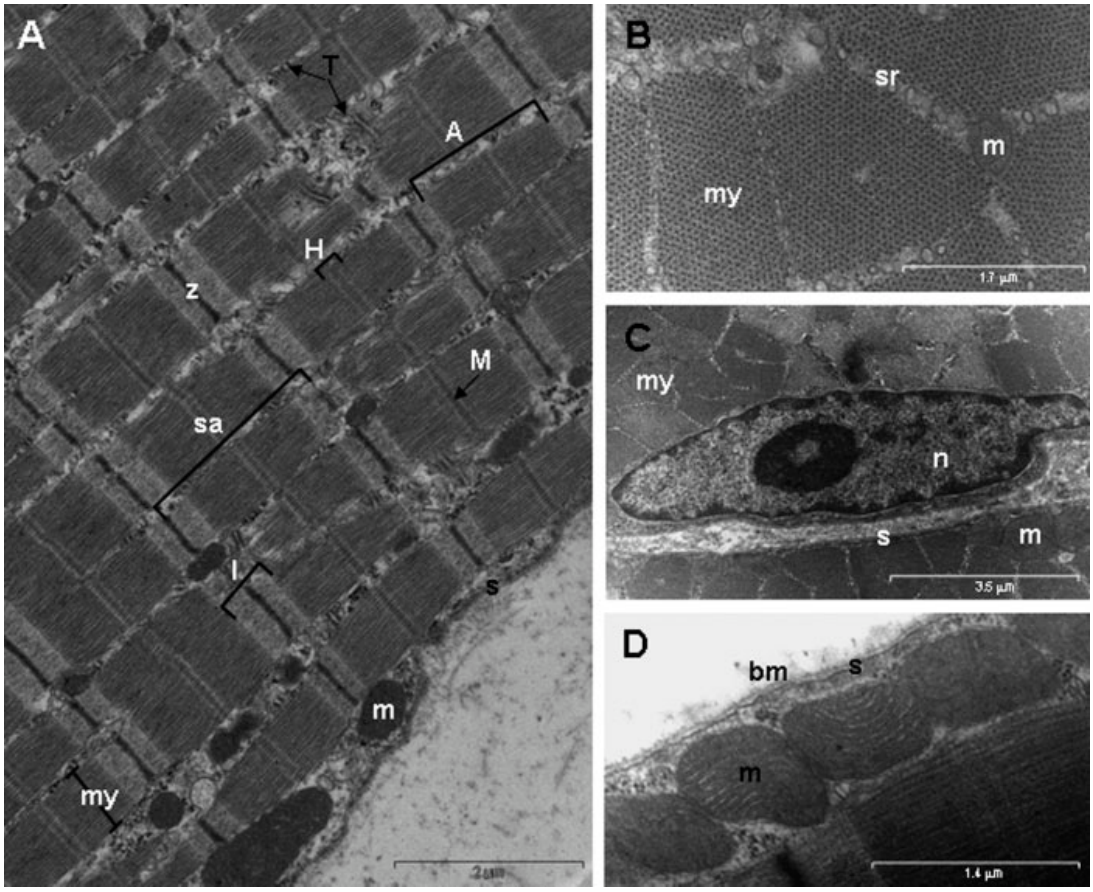

Fig. 3. General aspects of control TA muscles. (**A**, **D**) Longitudinal sections. (**B**, **C**) Transverse sections. (C–D) Note the nucleus (n) and the mitochondria (m) in intact myofibers (my). Sarcomere (sa), A-band (A), I-Band (I), Z-line (Z), H-band (H), M-line (M), T-tubule (T), sarcolemma(s), sarcoplasmic reticulum (sr), basement membrane (bm).

the natural Polybia-MPII mastoparan and the synthetic peptide was determined as being 1,613 Da and 1,613.5 Da, respectively.

Experimental Procedures

Mice were deeply anesthetized with a 1:1 mixture of ketamine chloride (Dopalen®, Vetbrands, 100 mg/kg of animal) and xylazine chloride (Anasedan®, Vetbrands, 10 mg/kg) (2 μL/mg body weight, i.p.). The right TA muscle was exposed, injected intramuscularly (i.m.) into the middle third with 0.25 μg/μL of mastoparan from *P. paulista* venom in a volume of 100 μL of physiological saline solution, after which the surgical wound was sutured. In a single stinging, a worker wasp is able to inject into victim ~30 μg of total venom (~1 μg/μL). From this amount 30% is constituted of MP (Palma, 2006), which means that 9 μg of MP is inoculated in each stinging at a 0.30 μg/μL rate concentration. The 0.25 μg/μL MP concentration used in this study was considered to be very close to the peptide concentration contained in the amount of venom inoculated by 1–3 sting(s) by wasp workers. Control animals were injected with saline solution only (sham group).

Transmission and Electron Microscopy

After 3 and 24 h, 3, 7, and 21 days (n = 6/period) of MP or saline injection the mice were anesthetized and the TA muscles were dissected. The muscles were sectioned in two halves at the point of MP injection. From one half, 1 mm fragments of TA were obtained and maintained in Karnovsky fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2) overnight, rinsed in 0.05 M PBS, postfixed for 2 h in 2% OsO_4, and rinsed again in 0.05 M PBS. The samples were dehydrated in acetone (30–100%) and embedded in Epon-Araldite resin mixture. Resin-blocked samples were trimmed perpendicular to the long axis of the muscle fibers and 60–70 nm thick ultrathin sections were collected on Formvar-coated copper slot grids and stained with 2% uranyl acetate in 0.05 M maleate buffer and lead citrate. All analyses were done with a Leo 902 Zeiss transmission electron microscope operated at 60 kV.

Light Microscopy

The other half of the TA muscle was fixed in 4% paraformaldeyde fixative overnight, after which the pieces were rinsed in 0.05 M PBS, dehydrated in graded ethanol series (70, 80, 95, and 100%), and embedded in

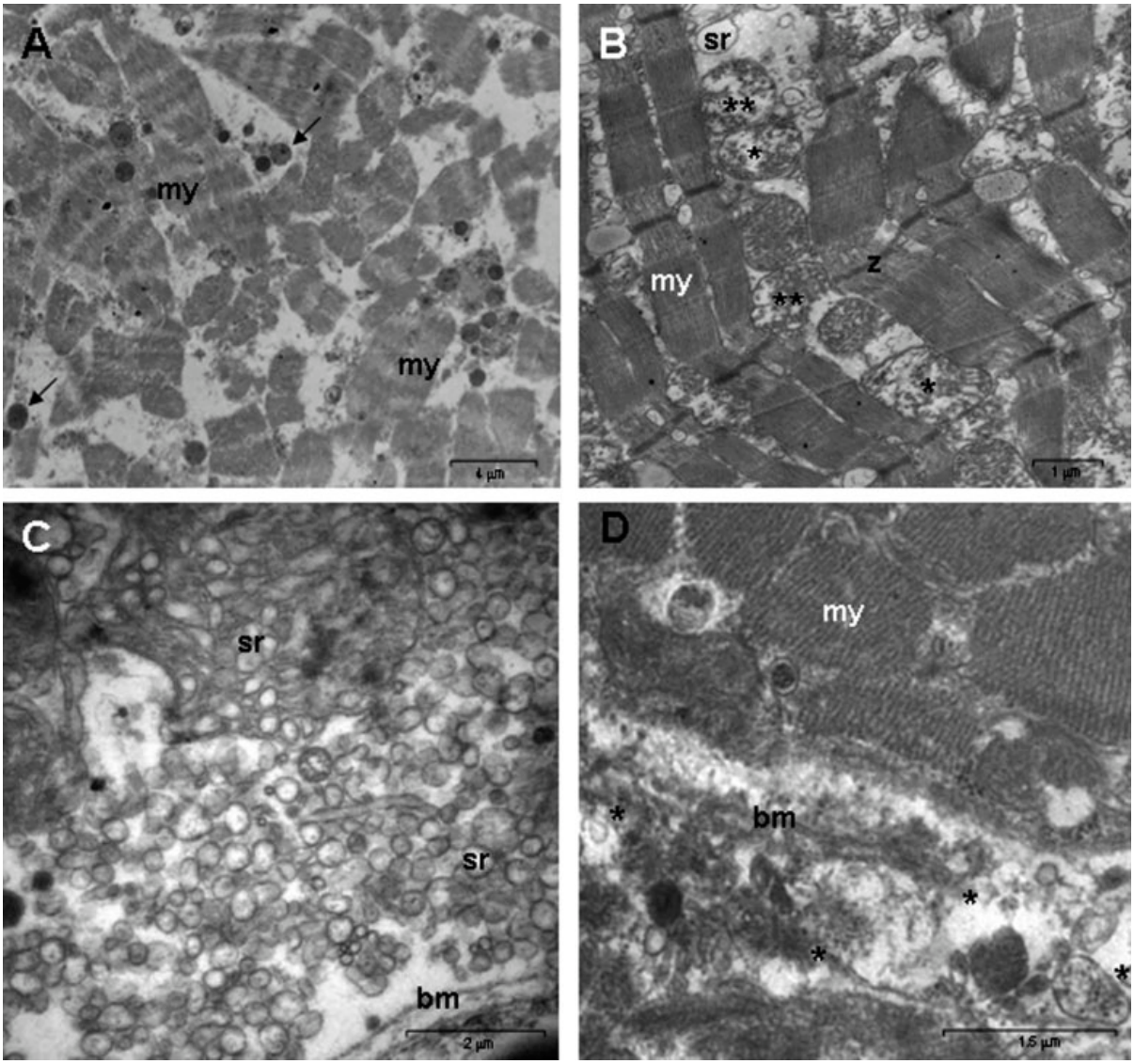

Fig. 4. Sections of TA muscle after 3 and 24 h after i.m. injection of Polybia-MPII mastoparan (0.25 µg/µL). (**A**) Several pieces of myofibrils irregularly oriented among which small electrondense mitochondria were scattered (arrows). (**B**) Detail of a fiber whose sarcolemma was not disrupted: the swelling of SR terminal cisternae (sr) and mitochodria distorts the myofibrils (my) orientation. Some mitochondria are involved by a single membrane (*) in others the mitochondrial envelope was ruptured (**). T-tubules are intact. (**C**) Detail of a fiber where sarcolemma was damaged: the sarcoplasmic reticulum (sr) was fragmentated into small vesicles, but the basement membrane (bm) was preserved. (**D**) Detail of two adjacent fibers with interrupted sarcolemma at the points marked with asterisks; myofibrils (my).

Leica historesin. Sections 5-µm thick were cut using a Leica RM 2035 microtome and stained with Toluidine Blue (TB) and Mallory's Trichrome (MT) for examination by light microscopy.

RESULTS

In the control muscles, injected with saline solution, the typical ultrastructure of normal skeletal muscle was maintained unchanged. Figure 3A shows paralleled myofibrils with sarcomeres exhibiting bands A and I in register and intact T-tubules. Longitudinal and cross sectioned myofibrils were typically separated by profiles of sarcoplasmic reticulum (SR) and mitochondria (Figs. 3A and 3B), and the nucleus was subsarcolemmal (Fig. 3C). Subsarcolemmal mitochondria were rich in normal looking cristae and the sarcolemma and surrounding basal lamina were continuous (Fig. 3D).

On the other hand, the envenomed groups presented structural changes which varied from slight to severe damage corresponding to different pathological states of fibers, depending on the period observed after i.m. injection. In all periods, the relative proportion of tissue with normal and altered morphology was variable. However, at 3 and 24 h post i.m. injection of MP, the altered area surpassed the unaltered, while from 3 to

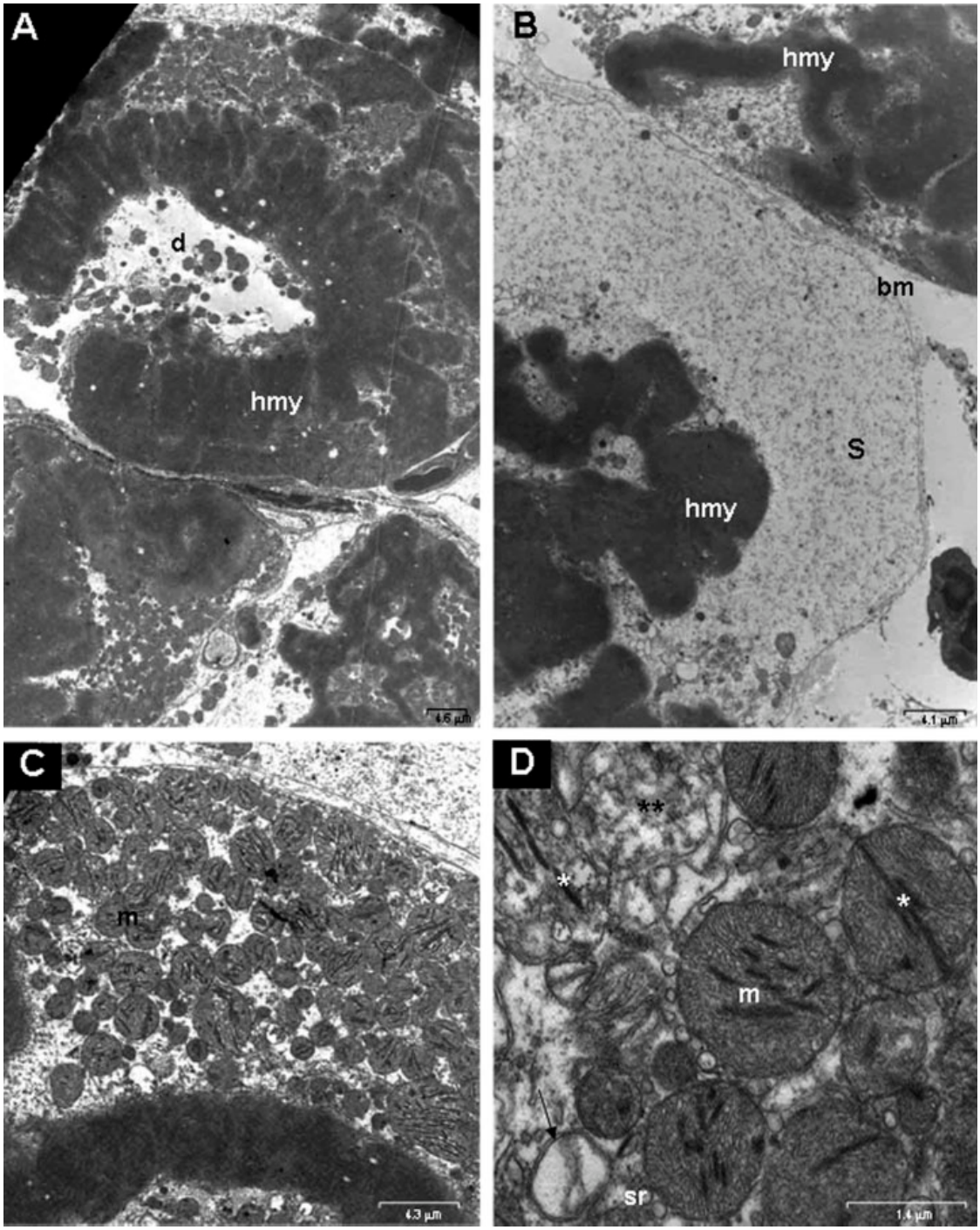

Fig. 5. (**A**–**D**) Damaged muscle fibers after 3 and 24 h of i.m. MP injection (0.25 μg/μL). (A–B) The micrograph show portions of five myofibers showing tortuous hypercondensation of myofibrils (hmy), pulverulent sarcoplasma (s), clusters of abnormal mitochondria (m), and the basement membrane (bm) surrounding the sarcolemma. A detail of a fiber with ruptured sarcolemma and δ-shaped lesion (d) is displayed in panel A. (C–D) Swollen mitochondria (m) with stacked electrondense cristae (*); or mitochondria disruption (**), sometimes devoid of cristae (arrow).

21 days the picture progressively reverted. Figures 4A and 4B (3 and 24 h) showed necrotic fibers, where myofibrils after hypercontraction underwent fragmentation or were displaced apart by clusters of abnormal mitochondria or membranous remnants of SR. The typical organization of sarcomeres in register was lost and Z line could be interrupted. The architecture of the SR originally displayed along the length of the myofibrils

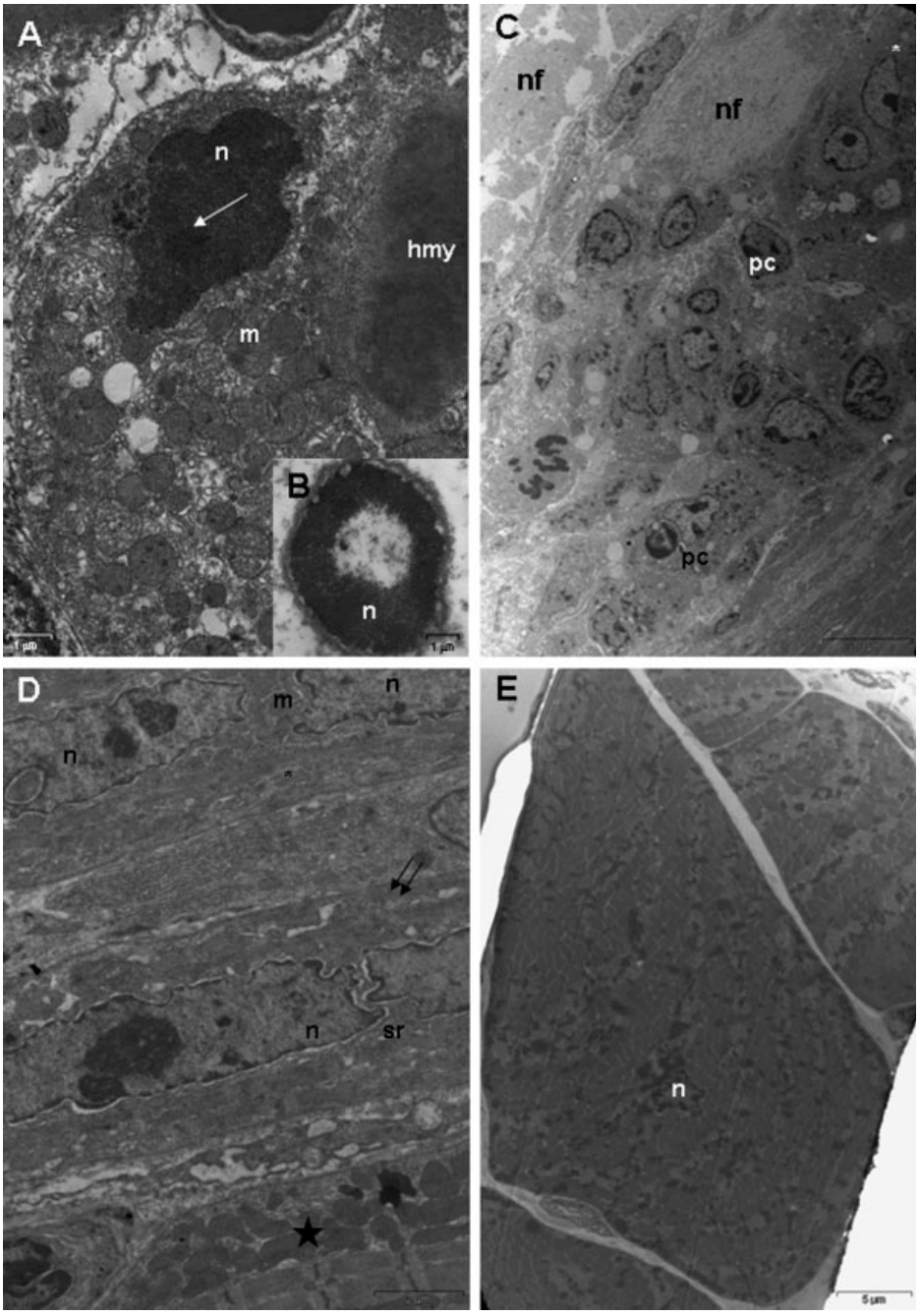

Fig. 6. (**A**, **B**) 3 and 24 h after i.m. injection of MP (0.25 μg/μL). (A) Apoptotic-like nucleus with very condensed chromatin where a nucleolus-like body is barely seen (arrow) of a fiber with hypercontracted and condensed myofibrils (hmy) and higly abnormal mitochondria (m). (B) Detail of part of an apoptotic-like nucleus (n) involved by the double nuclear envelope. (**C**) 3 days after i.m. MP injection, some longitudinal necrotic fibers (nf) and phagocytic cells (pc) are present. (**D**) After 7 days the regenerating fibers (*) showed rows of central elongated nuclei (n) and normal-looking mitochondria (m) and well-organized sarcoplasmic reticulum (sr). Sarcolemma and basement membrane appeared intact (double arrow). A fiber apparently not damaged present myofibrillar organization; oriented myofilaments and normal alignment of sarcomeres (star). (**E**) Detail of a cross-sectioned regenerative fiber 21 days after the treatment. Note the central nucleus (n).

could be disintegrated in numerous vesicles (Fig. 4C). Fibers with discontinuous sarcolemma maintained the basal lamina in position (Figs. 4C and 4D). On the other hand, cells with dilated SR cisternae were typical of fibers where sarcolemma was not discontinued. These cells appeared multivacuolated at light microscopy (not shown). Figures 5A, 5B, and 5C depict cross sections of necrotic fibers where different stages of hypercontraction and crumpled myofibrils resulted in large areas of denuded sarcoplasm sometimes only surrounded by the basal lamina. Displaced clusters of swollen electrondense mitochondria were seen. Mitochondrial alterations included changes in shape (round, elongated, or oval), variable amount, and electron density of cristae and matrix (compare Fig. 3D from controls with Figs. 5C and 5D of envenomed ones). In some swollen mitochondria cristae were absent or appeared as highly electron-dense stacked cristae. Furthermore, many mitochondria had only one membrane (Fig. 5D). In addition, the damaged fibers showed autophagic vacuoles in the subsarcolemmal space, including myelin-like figures, or were com-

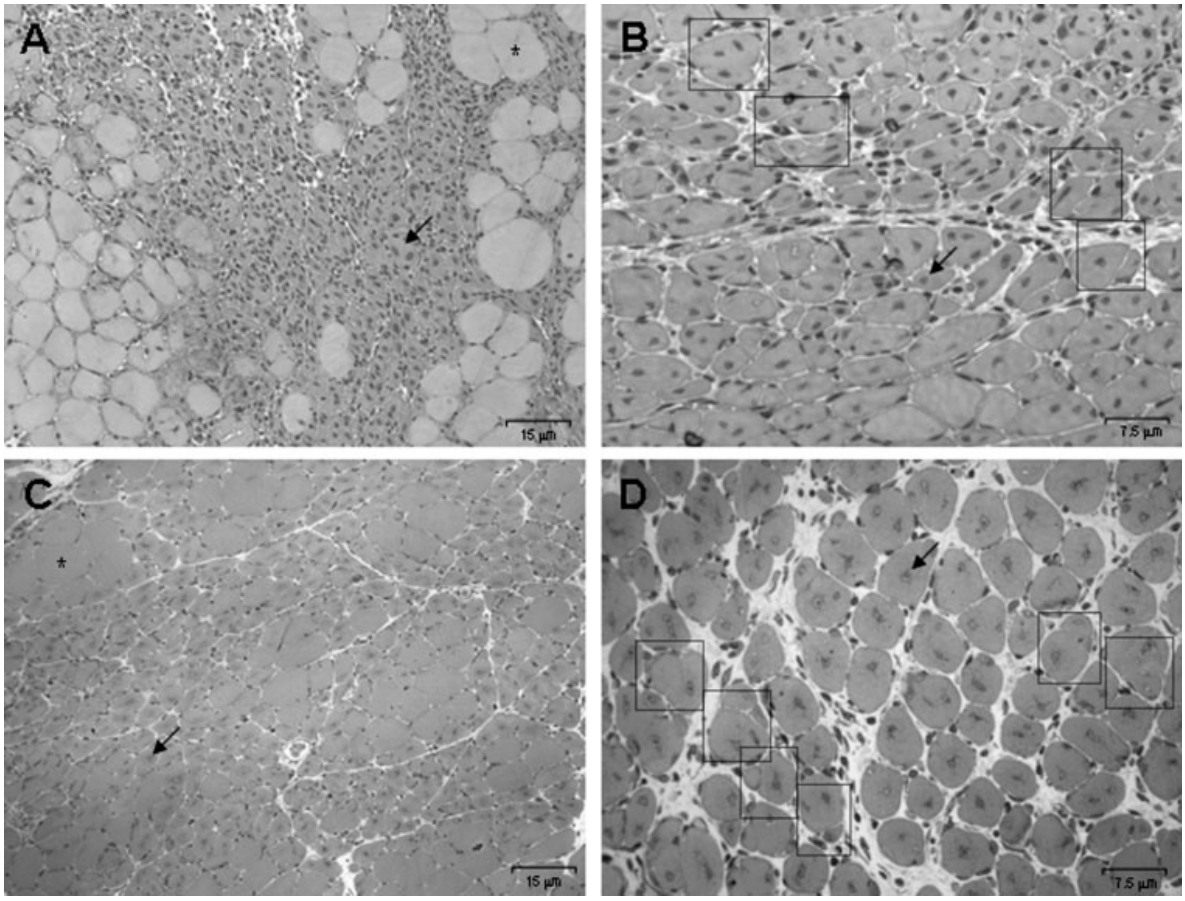

Fig. 7. TB light micrographs of MP-injected TA muscle displaying the tissue regeneration at day 7 (**A**, **B**) and day 21 (**C**, **D**). Regenerative fibers population increased markedly from day 3 (not shown) to day 7; myofibers with one or more central nuclei (arrows) presented a clear higher caliber at day 21 post-MP than at day 7. Split fibers with two or more small daughter fibers were common among the regenerative fibers (squares).

pletely devoid of any organelles (not shown). In some severely altered fibers with profound contractile apparatus disorganization, distorted apoptotic-like nuclei were viewed at 3 (Fig. 6A) and 24 h (Fig. 6B) after MP i.m. injection. Neutrophils and phagocytic inflammatory cells were present in the interstitium among the fibers (not shown).

Three days after MP injection although necrotic fibers and phagocytic cells still persisted (Fig. 6C), small regenerating myoblasts located within the basal lamina of necrotic cells start to appear indicating that regeneration was underway. One week (7 days) after envenoming, there were many regenerating cells with central nuclei, longitudinally oriented myofilaments, normal myofibrils distribution, alignment of sarcomeres, and normal-looking organelles. Sarcolemma and basal lamina were intact (Fig. 6D). Three weeks (21 days) after, the regenerative fibers reached almost the normal size, but in a number of them a small nucleus still remained in the center (Fig. 6E).

Light microscopy histological sections were analyzed just to follow the pathogenesis at each time point. A detailed description of the morphology of the muscle in each period was provided in a previous report (Rocha et al., 2007). However, an overview of the regeneration period of the TA muscle at day 7 (A and B) and day 21 (C and D) after i.m. injection of MP was included (Fig. 7). Figures 7B and 7D are higher magnifications of Figures 7A and 7C, respectively. Lower magnifications (Figs. 7A and 7C) allow comparison of the regenerating stages of fibers after 7 and 21 days of the injection. A higher magnification allows seeing that in both periods split fibers were interspersed among the other fibers (Figs. 7B and 7D). Muscle fiber splitting was absent in saline-injected muscle (not shown).

DISCUSSION

Our findings showed that Polybia-MPII mastoparan caused profound damage in the ultrastructure of the skeletal muscle fibers which seemed to have its onset through disturbances in sarcolemma. This is followed by fiber contractile apparatus structural disorganization and marked mitochondrial and smooth endoplasmic reticulum alteration. The ultrastructural findings add new insight to recent work, which using light microscopy, morphometry, and measurements of creatine kinase (CK) serum levels, showed that Polybia-MPII

from the *P. paulista* social wasp causes extensive myonecrosis (Rocha et al., 2007).

Among the components present in wasp venoms, MP responds for a vast repertoire of functions. All of them place cell membrane in the center of the discussion. Although no selective MP membrane receptors have been identified so far, among the pharmacological effects of this wasp peptide it has been of note its activation of G protein. G proteins belong to a family of proteins which is direct or indirectly involved in intracellular pathways regulating the function of virtually every organ and tissue. Studies report that G proteins can function as a signal transducer in several roles attributed to MP (Holler et al., 1999; Sukumar and Higashijima, 1992; Wakamatsu et al., 1992), including by activating selectively the phospholipase D_2 in cell membranes where the enzyme mediates exocytosis through GTP-binding proteins (G proteins) (Chahdi et al., 2003).

A series of studies have shown that the way MP molecule may interact with membrane phospholipids is by conformational changes of the molecule. Its amphiphilic nature allows that on binding with membrane phospholipid bilayer, an α-helical conformation is formed even in an aqueous milieu as the cytosolic. Such a conformational change is primarily due to the interaction between the aliphatic side chains of MP and the hydrophobic interior of phospholipid membrane (Higashijima et al., 1983; Marsh, 1996; Nomura et al., 2004; Ojcius and Young, 1991; Sansom, 1991; Wakamatsu et al., 1992). As a consequence, membrane physicochemical characteristics are perturbed and eventually pores can be formed (Arbuzova and Schwarz, 1996; Dempsey, 1990; Katsu et al., 1990; Mellor and Sansom, 1990) caused by considerable increase of permeability of lipid bilayer permeability for hydrophilic substances and rise of PLA_2 activity (Argiolas and Pisano, 1983), resulting in loss of its intrinsic properties as a membrane.

The confrontation of our ultrastructural findings and the MP pharmacological actions, described in literature, allows understand the consequences brought about by interactions established between the peptide and fiber plasma membrane here seen. In agreement with our previous concluding remarks (Rocha et al., 2007), the present findings point sarcolemma as a probable primordial target of MP. We raised the hypothesis that the disruption of sarcolemma was likely co-operated by the reported MP capacity of both perturbing Ca^{2+} mobilization (Longland et al., 1998, 1999) and impairing the protein kinase C (PCK) (Raynor et al., 1992). Our proposal is based on the fact that PCK mediated-phosphorylation of dystrophin is required for maintaining sarcolemma mechanical stability and its bind to myofibrillar cytoskeleton, a phenomenon which is Ca^{2+}-mediated (Luise et al., 1993; Senter et al., 1995). Moreover, the phosphorylation of dystrophin is activated by cAMP, cGMP, calcium, and calmodulin, and was inhibited by cAMP-dependent protein kinase peptide inhibitors, such as the mastoparan and heparin (Luise et al., 1993; Raynor et al., 1992).

One of the ensuing steps in fiber myonecrosis after rupture of the sarcolemma is the formation of δ-like lesions, where the onset of fiber lysis assumes the shape of a delta letter. As a consequence there is a secondary unspecific influx of calcium in the fiber, which can trigger Ca^{2+}-dependent endogenous proteases activation and degradation of cell components (Duncan, 1978), so contributing for cell damage. Some or all these events could be involved in the structural changes as reported here.

The current study showed that together with the rupture of sarcolemma and collapse of the myofibrilar contractile apparatus, the membrane of SR cisternae underwent fragmentation in numerous vesicles. Studies have shown that MP activates a 97 kDa rhyanodine receptor-binding protein identified as being a glycogen phosphorylase, and promotes Ca^{2+} release from SR (Hirata et al., 2003).

A synergic effect of the MP related to the increase of cytosolic Ca^{2+}, is induced by the potent SR-Ca^{2+}-ATPase (SERCA)-inhibiting effect exerted by the peptide (Longland et al., 1999). SERCA is responsible for reuptake of Ca^{2+} into SR cisternae during relaxation of the muscle fiber, coupled to ATP hydrolysis. On the other hand, mastoparan was shown to inhibit the affinity of the ATPase for Ca^{2+} and abolishes the cooperation of Ca^{2+} binding (Longland et al., 1998, 1999). These synergistic actions of MP, activating the release and inhibiting reuptake of Ca^{2+} causes overload of calcium a condition harmful to the cell and which could explain the hypercontraction of myofibrils observed in the present study.

Both mitochondria and SR are cell components directly involved in calcium buffering in skeletal muscle fibers. Mitochondria play an important role in maintaining a low sarcoplasmic Ca^{2+} concentration. Disturbances of Ca^{2+} overload affect the mitochondrial buffering role. On the other hand, a growing bulk of studies have proved MP as able to increase permeability of the inner mitochondrial membrane and induce opening of large pores, a phenomenon linked to cell Ca^{2+} overload and redox stress (Lemasters et al., 1998; Armstrong, 2006; Pfeiffer et al., 1995; Szabó and Zoratti, 1992; Zoratti and Szabó, 1994).

In this work, the ultrastructural evidences of disturbances of mitochondrial membrane permeability were shown by the swollen electronlucent mitochondria and collapsed closely-applied electron-dense cristae exhibited. Mitochondrial pore transition has been associated with the triggering of apoptotic cell death. Apoptotic-looking nuclei were seen in the current study with MP and in studies with the crude venom (Paes-Oliveira et al., 1998, 2000). We suggest that the mitochondrial alterations of size, shape, matrix and cristae seen after 3 and 24 h of MP envenoming could reflect the changes in mitochondrial permeability (Berman et al., 2000). A correlation between such mitochondrial structural changes, the appearance of apoptotic-looking nuclei and impairment of cell Ca^{2+} homeostasis needs further substantiation.

Despite the strong myotoxicity exhibited by mastoparan, the changes appear not to incapacitate the ability of satellite cells responsible for regeneration of fibers to be activated. Unpublished results of our laboratory showed that PLA_2 from *P. paulista* venom, although significantly less myotoxic than Polybia-MPII mastoparan does not preserve the rate of regeneration as that exhibited by mastoparan. This is another interesting characteristic of MP to be explored. Our findings

showed also that split fibers appeared among the regenerating ones. Split fibers have ultimately been considered as signal of defective regeneration, although other plausible hypotheses such as mechanism for reducing distance for oxygen and metabolites diffusion in hypertrophic fibers, replacement of necrotic fibers by activation of satellite cells, or activation of satellite cells even in the absence of fiber necrosis, and signal of fibers undergoing degenerative changes. Other alternate explanation for this phenomenon is that fibers splitting can be related to effects of denervation and reinnervation (see Eriksson et al., 2006).

In conclusion, our results show a hitherto based-ultrastructure study of MP effects. The study showed that Polybia-MPII mastoparan damages the plasma membrane of myofibers but leaves basal lamina, severely affects the structure of SR and mitochondria, affects sarcomere organization, as well induces splitting of fibers. Probably, satellite cells are not affected, allowing rapid and reproducible muscle regeneration. The study of the actual mechanism of MP in muscle fibers will contribute to clarify all these questions.

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Capítulo 3

The neurotoxicological effects of mastoparan Polybia-MPII at the murine neuromuscular junction: an ultrastructural and immunocytochemical study

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The neurotoxicological effects of mastoparan Polybia-MPII at the murine neuromuscular junction: an ultrastructural and immunocytochemical study

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Abstract

Polybia-MPII (INWLKLGKMVIDAL-NH_2), a mastoparan isolated from the crude venom of the swarming wasp *Polybia paulista*, was injected into the left hind limb of Swiss white mice. Between 3 hours and 21 days later the mice were killed and the soleus muscles from both hind limbs were removed. Sections of the muscles were made for transmission electron microscopy and immunocytochemistry. Transmission electron microscopy revealed that exposure to Polybia-MPII caused no significant structural damage to the plasma membrane of the terminal boutons and mitochondria were indistinguishable from those in normal, control boutons but in many cases the volume fraction occupied by synaptic vesicles was greatly reduced. In control muscles 99% of motor end plates identified by positive labeling of ACh receptors by TRITC-α-bungarotoxin co-labeled with anti-synaptophysin Ab, but this figure fell by 30% in muscles exposed to the toxin. These changes were transient. They were maximal at 6 hours and fully reversed by 3 days. At no time was axonal labeling with anti-neurofilament antibodies affected by exposure to Polybia-MPII.

Keywords: mastoparan, neuromuscular junction, Polybia-MPII, synaptic vesicles, wasp.

Introduction

Swarming wasps are widespread in the tropical and subtropical world and stings by these insects constitute a significant medical emergency. Brazil is home to more than 200 species of wasps and more than 10,000 incidents are recorded each year; 2,000 cases of severe stings per year are presented to hospital clinics in São Paulo State (Fig.1a) out of total population of 39.8 million persons. One species of wasp, *Polybia paulista* (Vespoidea, Vespidae, Polistinae) (Fig.1b), is responsible for the majority of incidents. The signs and symptoms of stings by tropical wasps are, most commonly, purely local and include urticaria, pain and inflammation. Such stings can be treated symptomatically. Serious systemic envenoming may also involve cardiovascular and respiratory systems (bradycardia, cardiac arrhythmias, angina, hypotension, respiratory distress) and rhabdomyolysis and peripheral motor and sensory neurotoxic syndromes are not uncommon (Antonicelli et al. 2002; Steen et al. 2005). The local and systemic signs and

symptoms of severe envenoming are similar in all large vertebrates (Fitzgerald and Flood 2006).

The crude venom of *P. paulista* has been shown to cause a failure of the mechanical response of the indirectly stimulated diaphragm muscle of the mouse (Paes-Oliveira et al. 2000). The response was considered by the authors primarily to reflect degenerative changes in the skeletal muscle fibres but a transient increase in the frequency of the miniature end-plate potentials (mepps) at early stages of poisoning and the presence of omega (Ω)-shaped indentations in the plasma membrane of terminal endings suggested the possible existence of a neurotoxic component in the venom causing an enhanced exocytosis and a reduction in synaptic vesicle recycling.

The venoms of wasps are complexes mixtures of phospholipases, hyaluronidases, acid phosphatases, proteases, nucleases and numerous small polypeptides many of which remain poorly characterized. Among the better known are several enzymes with hyaluronidase activity, haemolytic phospholipases, polybitoxins and mastoparans (Nakajima et al. 1985; Piek. 1990; Hoffman 1996, 2006; Oliveira and Palma 1998; de Souza et al. 2004; Santos et al. 2007). The mastoparans comprise a large family of small toxic polypeptides many of which are secretogogues (see, for example, Hirai et al.1979; Kurihara et al. 1986; Yokokawa et al. 1989; Wilson 1989; Joyce-Brady et al. 1991; Komatsu et al. 1992, 1993; Jones et al, 1993; Misonou et al. 1997; Seebeck et al. 2001; Bensimon et al. 2004). Thirty percent of the venom of *P. paulista* comprises the mastoparan Polybia-MPII (INWLKLGKMVIDAL-NH_2) (M.S. Palma, personal communication). It is myotoxic (Rocha et al. 2007; 2008) and it seemed reasonable to ask whether Polybia-MPII was also neurotoxic. In this communication we describe our observations on the neurotoxic activity of Polybia-MPII and we discuss the possibility that the toxin contributes significantly to the systemic signs and symptoms of severe envenoming by *P. paulista* in the human.

Material and methods

Animals

Thirty seven adult male Swiss white mice (~30g) obtained from Charles River Animal Supplies (Margate, UK) were maintained under veterinary supervision, according

to the requirements of the Committee on Ethics (Newcastle University) and the Animals (Scientific Procedures) Act 1986. They had free access to food and water.

Muscles

The soleus muscles were used exclusively in this study because carefully made subcutaneous inoculation can ensure that the entire muscle is readily exposed to experimental agents and the muscle can be removed intact from tendon of origin to tendon of insertion.

Toxin and reagents

Polybia-MPII mastoparan was synthesized according to the protocol described by de Rocha et al (2007). FITC and TRITC-conjugated α-bungarotoxin was obtained from Molecular Probes Inc. Rabbit anti-synaptophysin (cat. RB1461) was purchased from Neomarkers. Chicken anti-neurofilament protein (cat. AB5539) was purchased from Chemicon International. FITC-conjugated donkey anti-chicken IgG (cat.703 095 155) was purchased from Jacksons ImmunoResearch Inc. FITC-conjugated swine anti-rabbit IgG (cat.F0205), TRITC-conjugated swine anti-rabbit IgG (cat. R0156) and FITC-conjugated goat anti-mouse IgG (cat. F0479) were obtained from DAKO. All secondary antibodies were incubated with rat serum and centrifuged to yield a clear supernatant before use. Other reagents of analar grade were obtained from regular commercial supplies.

Experimental procedures

Polybia-MPII mastoparan (25 μg in 100 μl 0.9% w/v NaCl) was injected sub-cutaneously into the antero-lateral aspect of the right hind limb to bathe the underlying soleus muscle. The left hind limb was used as the control muscle. In three cases the left limb was injected with 100 μl 0.9% w/v NaCl. There was no observable difference between the injected and the non-injected control muscles and they are not further differentiated. Between four and six animals were humanely killed at three, six and 24 hours, and 3, 7 and 17-21 days after inoculation. Both left and right soleus muscles were removed, pinned onto dental wax at ~1.2x resting length and processed for transmission electron microscopy (TEM) and immunohistochemistry (ICC).

Transmission Electron Microscopy (TEM)

Small blocks of soleus muscle presumed to contain motor end plates were fixed in 2% glutaraldehyde in Sorenson's phosphate buffer for 1 hour, trimmed into small segments of 1x1x2 mm and returned to the fixative for a further 24 hours. They were then rinsed in Sorenson's phosphate buffer 2 times in 15 min, post-fixed in 1% OsO_4 (in buffer) for 1 h, rinsed again in the same buffer overnight, dehydrated in a ascending concentrations of acetone (25% to 100%) and embedded in TAAB epoxy resin. Sections were cut at a thickness of 60-70 μm (silvery gold in colour) were cut from blocks of control muscles and from muscles 3 hr and 24 hr after exposure to Polybia-MPII mastoparan for the identification and examination of nerve terminals. Between 9 and 24 terminal boutons were examined from each time point.

Immunocytochemistry (ICC)

The medial third of the soleus muscle was mounted onto a strip of filter paper, supported with tissue-tek OCT embedding medium and frozen in isopentane cooled in liquid nitrogen. Cryosections (6 μm for TS and 20 μm for LS) were mounted on subbed slides, permeabilized in cold (-20^{o} C) ethanol and methanol, and 0.1 % Triton X-100 in phosphate buffered saline (PBS) (10 min each at room temperature) and rinsed with PBS. The sections were then incubated overnight in a closed moist chamber at 4^{o}C with appropriate primary antibodies diluted in PBS containing 0.1 M lysine and 3 % BSA to a final concentration of 1/100. Sections were then allowed to return to room temperature, washed 2 times in 30 min in PBS before being counter labeled (see below).

The colocalisation of junctional acetylcholine (ACh) receptors and synaptophysin (a specific label for synaptic vesicles) was studied on transverse sections. Permeabilized sections were incubated with anti-synaptophysin primary antibodies overnight (see above). The following day, slides were allowed to return to room temperature, washed in PBS 2 times in 15 min and incubated with a combination of the swine-anti rabbit secondary antibody and FITC-conjugated-αBTX for 2 h at room temperature and then mounted in Vectashield®. Some control sections were treated as described above, but the primary antibody was omitted.

To study the organization of the terminal innervation, longitudinal sections were labeled overnight with a combination of anti-synaptophysin and anti-neurofilament primary antibodies and then with a combination of the appropriate secondary antibodies and FITC,-conjugated-αBTX (as described above).The patterns of terminal innervation were defined as simple, when the postsynaptic region, characterized by a discrete cluster of ACh receptors, was innervated by a single axon; complex when the postsynaptic component was formed of two or more discrete but closely associated clusters of ACh receptors and when a single axon branched to innervate those clusters of receptors, and multiple when a single muscle fibre was innervated by more than one axon. Evidence of pre- and post junctional sprouting was also documented (see, for example, Dixon and Harris, 1999; Harris, 2006).

In all studies involving ICC ten slides each containing four sections were prepared and labeled. Three slides were randomly selected from each group of ten and all four sections were counted. The percentage of synaptic vesicles labeled with synaptophysin in relation to α-BTX labeled receptor was calculated as follow:

$$\% \text{ SV} = \frac{\text{No of synaptophysin labeled SV}}{\text{No of } \alpha\text{-BTX labeled receptor}}$$

On average, 13 labeled end-plates were identified on each section examined.

Histology

Transverse and longitudinal cryosections of muscle tissue were mounted on subbed slides, stained with haematoxylin and counterstained with eosin.

Morphometry and Statistical analysis

For the estimation of the volume fraction of a terminal bouton occupied by synaptic vesicles a grid of points was superimposed on each image of a bouton. The cross-sectional area of each terminal bouton and the volume occupied by the synaptic vesicles were calculated. All numerical data are expressed as mean ± standard deviation (SD) and were analyzed using GraphPad InStat version 3.0. The statistical significance of differences

among groups was determined by one-way ANOVA followed by the post-hoc test of Bonferroni for multiple comparisons. A p value <0.05 indicated statistical significance.

Results

Animal behaviour

Animals injected with Polybia-MPII showed no loss of exploratory behaviour, general mobility or general awareness. They exhibited no signs of lachrymation, salivation or rhinorrhea, or of gastrointestinal disturbance. No paralysis of the inoculated limb or mortality was observed. The animals tolerated handling and palpation of the limbs. They ate and drank freely.

Muscle pathology

Muscle fibres in control muscles were regular in size and outline and evenly stained with haematoxylin and eosin. Muscles exposed to Polybia-MPII were oedematous and pale and the extra-vascular spaces were often filled with inflammatory cells and erythrocytes. Between 3 and 24 hours vacuolated, swollen and hyaline fibres were commonly seen and many of the fibres were frankly necrotic. Three days after the inoculation of toxin, the inflammatory response had subsided and small, centrally nucleated fibres were common. Thereafter there was a progressive increase in the diameter of the regenerating muscle fibres, easily recognized by the continuing central location of their nuclei. Muscle fibre damage was extensive in all muscles examined, involving approximately 40% of the muscle fibres in the soleus muscles. They were identical in all respects to those described by Rocha et al (2007, 2008) and are not illustrated here.

Transmission Electron Microscopy (TEM)

Images of control neuromuscular junctions comprised between 1-4 terminal boutons, each sitting in a clearly defined, deeply folded post-synaptic trough in the muscle fibre membrane. The boutons were filled with synaptic vesicles and mitochondria and were capped by the processes of a terminal Schwann cell (Fig.2A). Neuromuscular junctions in the muscles of animals exposed to Polybia-MPII were similar to controls (Fig.2B-F) but

many exhibited a clear reduction in the density of synaptic vesicles in the terminal bouton (Fig.2C-D). This apparent depletion of vesicles was confirmed by morphometry, the volume fraction occupied by synaptic vesicles declining significantly from 33 ± 23.3% in control muscles to 19 ± 10.2% in muscles exposed to Polybia-MPII. There was also clear evidence of the shrinking of the terminal boutons, mean cross sectional area falling from 5.8 ± 3.53 μm^2 in boutons in control muscles to 3.1 ± 1.87 μm^2 in boutons in muscles exposed to Polybia-MPII. The morphometric data are summarized in Table 1. There were no obvious signs (Ω)-shaped indentations in the plasma membrane of the boutons, no significant mitochondrial damage and no structural damage to the axolemma of the terminal bouton.

Immunocytochemical Labeling (ICC)

Transverse sections of control muscles labeled positively for junctional ACh receptors and 99 ± 0.7% of identified junctions were counter-labeled with the anti-synaptophysin antibody. There was a significant reduction in the number of identified junctions counter-labeled with anti-synaptophysin in muscles exposed to Polybia-MPII. The peak loss of labeling occurred at 6 hours when 30 ± 4.9% of identified junctions failed to label with anti-synaptophysin. The loss of labeling was transient and by 3 days labeling was seen at 95 ± 3.6% of identified junctions, a level of labeling that remained constant at least until 21 days. The data are summarized in Figs. 4 and 5A.

Longitudinal sections of the muscles were labeled with TRITC-conjugated-αBTX and counter-labeled with a combination of anti-synaptophysin and anti-neurofilament antibodies. The images obtained showed a selective loss of the terminal labeling but intact labeling of axonal neurofilament protein. The loss of terminal labeling was particularly obvious at 6 hours (Figs.5B and 6). There was no difference in the pattern of labeling between control muscles and muscles exposed to Polybia-MPII (Table 1).

Discussion

Stings by arthropods are very common and cause much pain and discomfort to very large numbers of victims. The basic biology underlying the respiratory and cardiovascular problems associated with serious clinical envenoming have been extensively studied

(Golden 2007) but the rarer problems of myo- and neurotoxicity have generated much less interest.

The major component of the venom of *P. paulista* is a small, myotoxic mastoparan Polybia-MPII. We have now shown that Polybia-MPII is also neurotoxic, with clear evidence that its primary site of action is the terminal bouton of the motor axon. The pathological changes we observed were restricted to the most distal parts of the motor axon. Ultrastructural and morphometric studies of terminal boutons confirmed that Polybia-MPII caused a statistically significant reduction in the volume fraction of the terminal boutons occupied by synaptic vesicles. Apart from a reduction in cross-sectional area there was no evidence of structural damage to the plasma membrane of the nerve terminal or to mitochondria in the boutons. The reduction in the number of synapses labeling positively for synaptophysin was consistent with the morphological data.

The mastoparans are secretogogues, stimulating, for example, the exocytosis of inflammatory mediators from mast cells (Hirai et al. 1979), insulin and GABA from pancreatic beta-cells and insulin secreting cell-lines (Yokokawa et al. 1989; Hillarie-Buys et al. 1992; Jones et al. 1993; Komatsu et al. 1992; Ohara-Imaizumi et al. 2001). We suggest that the loss of synaptophysin labeling represents the enhanced exocytosis of ACh vesicles from the terminal boutons. The loss of synaptophysin labeling was transient and was fully reversed by 3 days. This is consistent with the rate of recovery of synaptic function following exposure to other toxins that cause a structural or functional disruption of neuromuscular transmission limited to the terminal axon and bouton (Dixon and Harris 1999; Harris 2006) and which may be ascribed to the very rapid upregulation of the production and transport of synapse specific proteins and synaptic vesicles in damaged neurons and motor axons (Roos and Kelly 2000).

The primary mechanism of the myo- and neurotoxic action of Polybia-MPII is not clear. Like other mastoparans, Polybia-MPII is a tryptophan containing polypeptide and it is oriented in such a way that the hydrophobic and hydrophilic residues are segregated to different faces of the molecule (Rocha et al. unpublished). We suspect that the molecule forms a carpet on lipid bilayers, anchored by the tryptophan residue and orientated such that the hydrophobic residues interact with the lipid heads of the outer lipids thus disrupting lipid packing and creating a mechanism whereby ionic leakage and the opening of ion

channels in the plasma membrane cell could lead to a depolarization of the cell (Mellor and Sansom 1990; Whiles et al. 2001) and an increase in intracellular calcium (Choi et a. 1992; Eddlestone et al. 1995).

Despite the clear demonstration the Polybia-MPII was both myo- and neurotoxic it is important to note that the animals exhibited no signs at all of discomfort or weakness to experienced small animal veterinarians and technical staff. The absence of behavioural signs of neuromuscular weakness in mice with clear cellular evidence of neuro-and myotoxicity is of some interest. We inoculated 25 μg of the toxin into each mouse. The average yield of crude venom from a single specimen of *P. paulista* is 30 μg of which 10 μg is Polybia-MPII (MS Palma, personal communication). Thus we were using a dose equivalent to that which would be inoculated by 2-3 wasps. If Polybia-MPII is responsible for the rhabdomyolysis and neurotoxic signs sometimes seen in human subjects (at a body mass of 60 kg) the equivalent dose Polybia-MPII would require multiple stings from between 4000 and 6000 wasps. There are no reported cases of multiple stings from this wasp. It seems likely that rhabdomyolysis and neurotoxicity represent rare idiosynchratic responses to envenoming.

In conclusion, there can be little doubt that the mastoparan Polybia-MPII is myotoxic and that it has some neurotoxic activity. There was reduction in the volume density of synaptic vesicle and reduction of the cross-sectional area of the nerve bouton, but any significant breakdown of the nerve terminal even at excessively high concentrations was found. At least in part, most of the myotoxic and neurotoxic effects of the crude venom can be attributed to mastoparan action. In terms of human toxicity it seems highly unlikely that the neurotoxic activity of the toxin could cause significant clinical difficulty.

Acknowledgements

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Table 1: Innervation patterns on soleus skeletal muscle after Polybia-MPII injection.

	Number of fibres						
Innervation pattern	Control	3 hours	6 hours	24 hours	3 days	7 days	17 days
Simple	58	30	40	40	47	46	30
Complex	-	-	-	-	-	-	1
Multiple	-	-	-	-	-	-	-

FIGURE 1

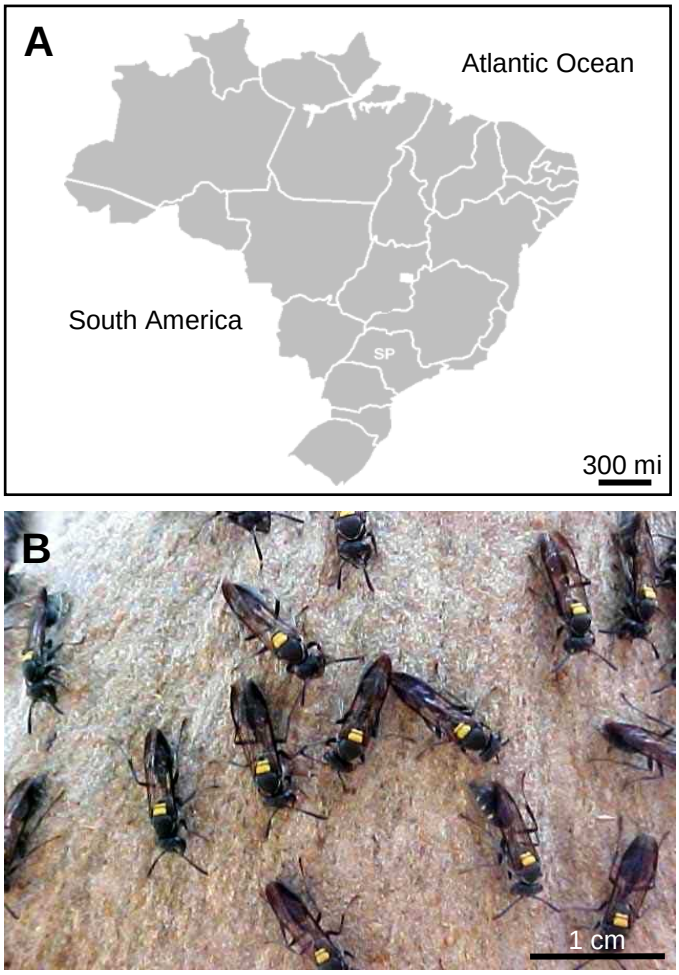

Figure 1: (A) Brazil and São Paulo State. Scale bar = 300 miles. **(B)** *Polybia paulista* wasps outside their nest. Scale bar = 1 cm.

FIGURE 2

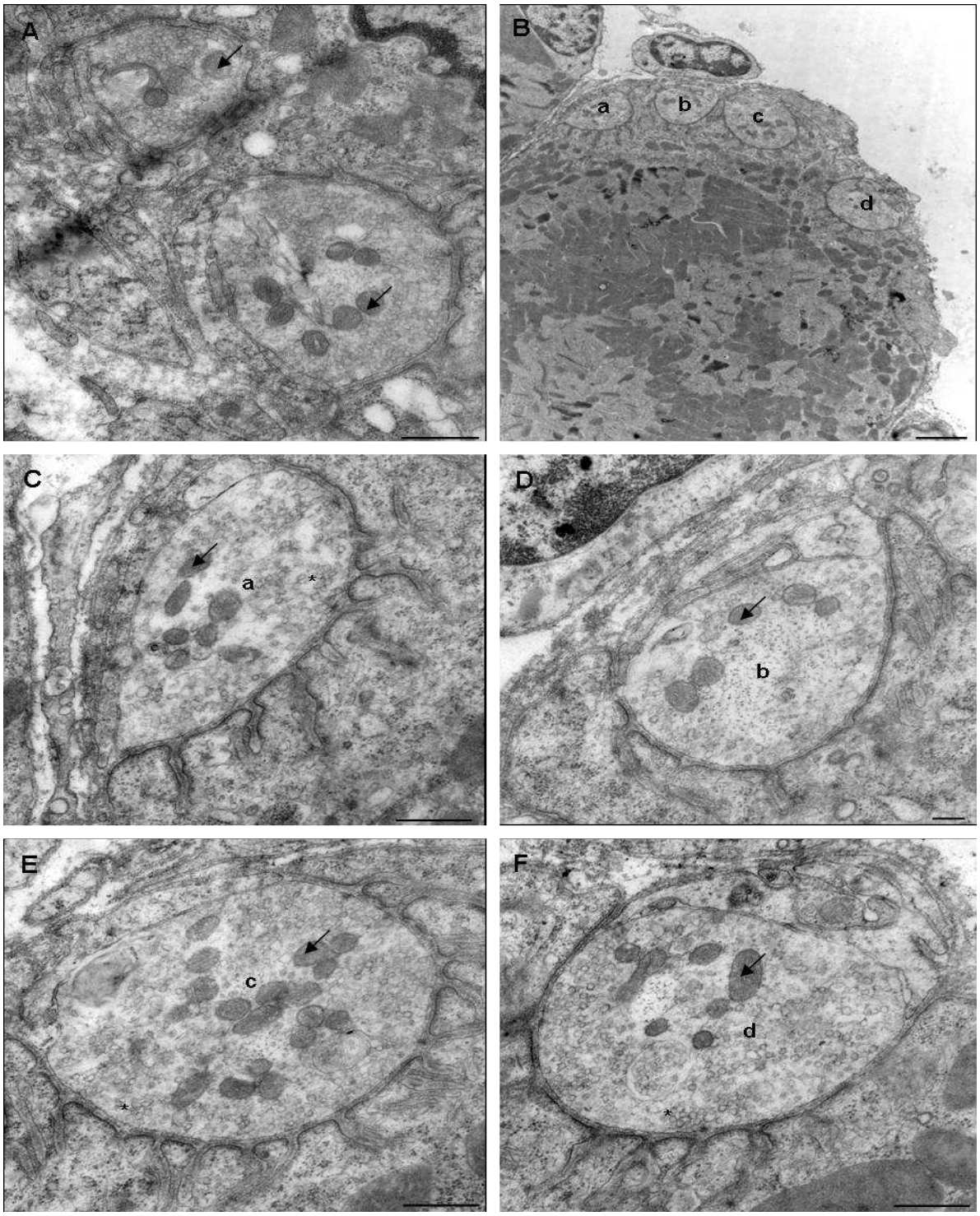

Figure 2: Images of neuromuscular junctions of control muscles **(A)** and 24 hour after the inoculation of Polybia-MPII **(B-F)**. In B the 4 boutons are signaled as **a**, **b**, **c** and **d** and are detailed in **C**, **D**, **E** and **F**, respectively. Note the depletion of synaptic vesicles (*) from the terminal bouton shown in **D**. Exposure to Polybia-MPII resulted in no observable damage to either the plasma membrane of the terminal bouton or to mitochondria (arrows) in the motor nerve terminal. Scale bar **(A, C-F)** = 500 nm; **(B)** = 2 μm.

FIGURE 3

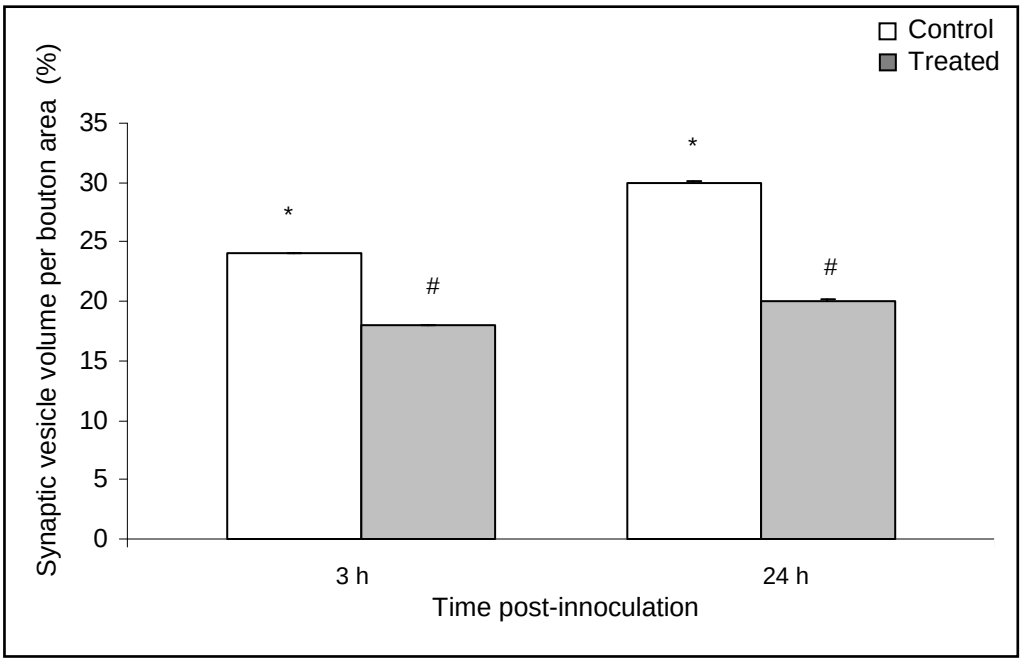

Figure 3: The fall in the volume fraction of terminal boutons occupied by synaptic vesicles 3 and 24 hours after the inoculation of Polybia-MPII. Significant differences were observed between controls (*) and treated (#) groups after 3 and 24 hours of MP-injection, ($p < 0.05$).

FIGURE 4

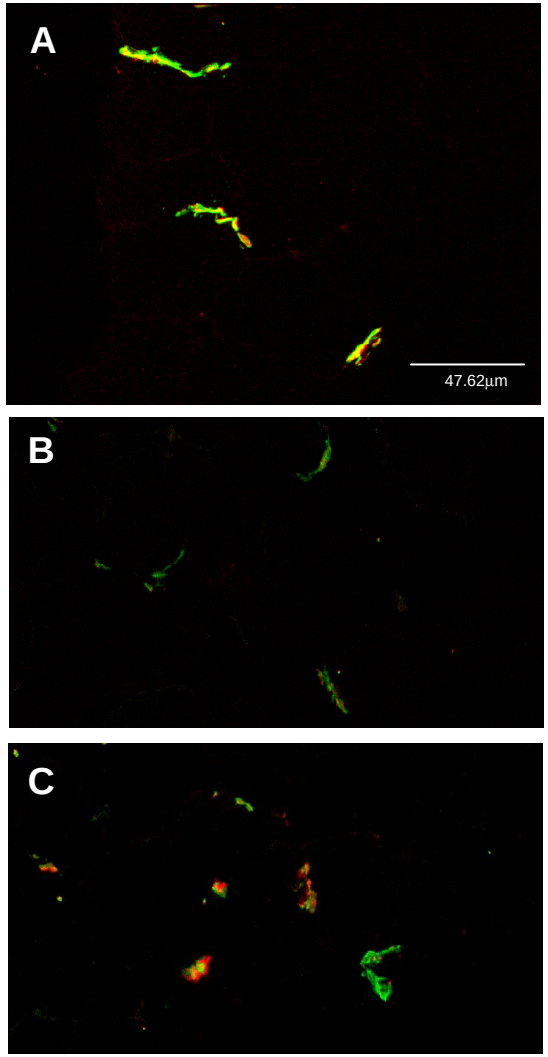

Figure 4: Transverse sections of control muscles **(A)** and 24 hour after the inoculation of Polybia-MPII **(B, C)**. In each case the sections were labeled with FITC-conjugated α-bungarotoxin (green) and counter-labeled with TRITC-conjugated anti-synaptophysin (red). Note the loss of immunolabelling with anti-synaptophysin in B and partial loss in C. Scale bar = 47.62µm.

FIGURE 5

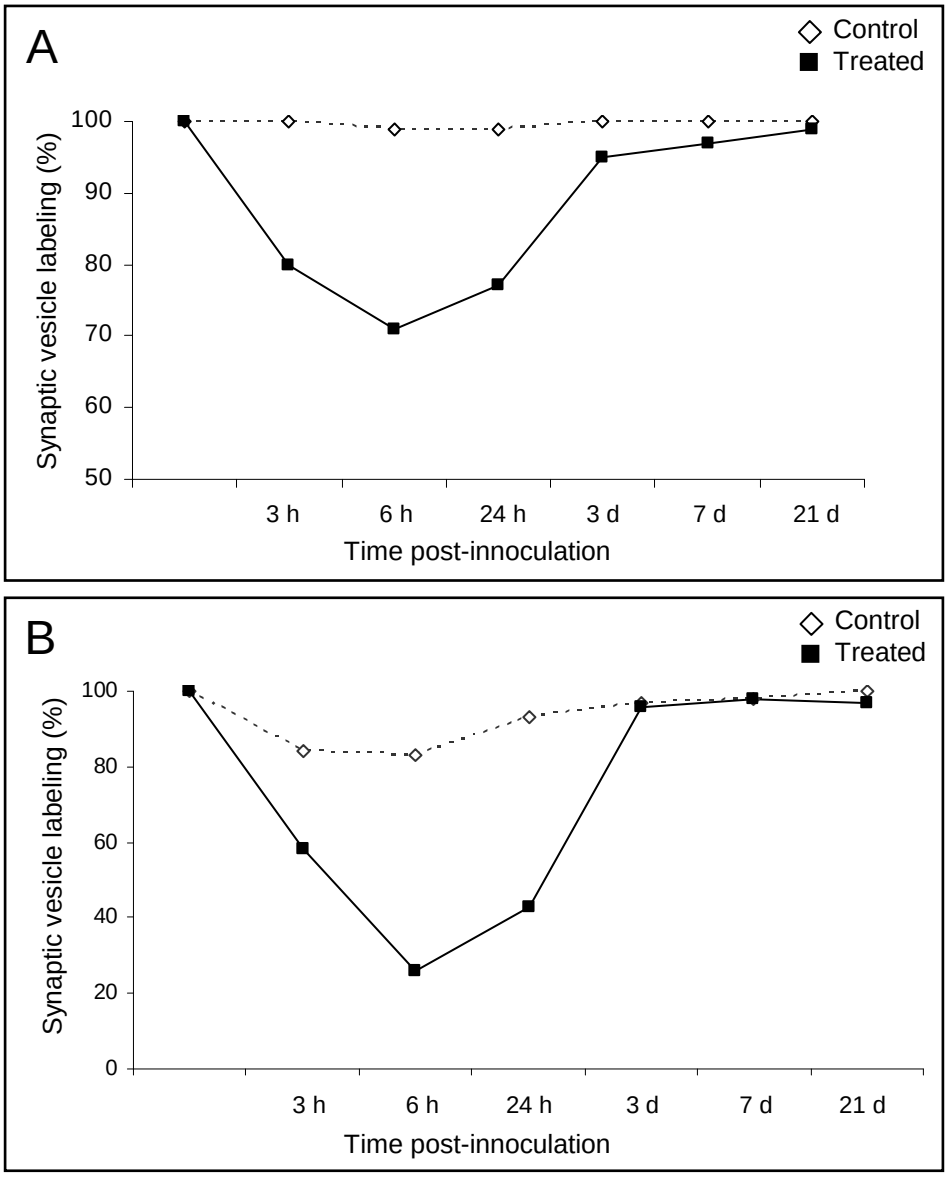

Figure 5: In both transverse **(A)** and longitudinal sections **(B)** of control muscles (◊) there was consistent immunolabeling of synaptophysin at end-plates identified by the co-labeling of ACh receptors by α-bungarotoxin. In muscles exposed to Polybia-MPII (■) there was a transient loss of immunolabeling by anti-synaptophysin antibodies.

FIGURE 6

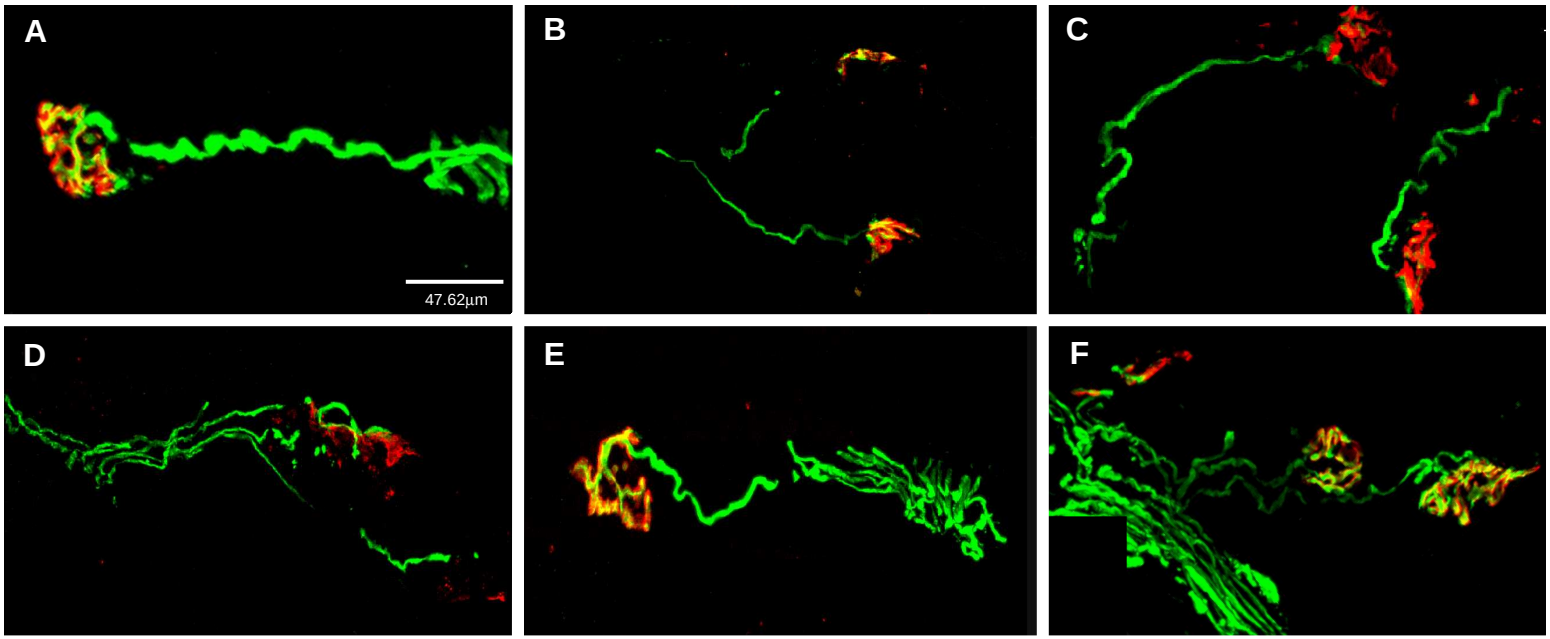

Figure 6: Longitudinal sections of control muscle **(A)** and at various times after the inoculation of Polybia-MPII **(B-F)**. ACh receptors were labeled with TRITC-conjugated α-bungarotoxin (red) and sections were co-labeled with a 1:1 mixture of anti-synaptophysin and anti-neurofilament followed by appropriate FITC-conjugated 2 antibodies (green). Note the loss of synaptophysin labeling from the intra-terminal spouts of the motor axon at 6 and 24 hours **(C, D)** and the restoration of labeling at 3 **(E)** and 21 days **(F)**. Neurofilaments appear to have remained intact. Scale bar = 47.62µm.

Capítulo 4

Analysis of cellular events associated with mastoparan-induced apoptosis on mouse tibial anterior muscle fibers

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Analysis of cellular events associated with mastoparan-induced apoptosis on mouse tibial anterior muscle fibres

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Abstract

Mastoparan first described as inducer of mast cell granules exocytosis has been implicated in many essential mechanisms of cell function. In skeletal muscle tissue the best characterization of mastoparan was induction of myonecrosis. Here, we investigate in different time points whether the mastoparan Polybia-MPII from *Polybia paulista* wasp venom promotes endonuclease and caspase 3 and 9 activation as well as inflammatory events accompanying these effects on mouse tibial anterior muscle. Through immunohistochemistry and immunoblotting, it was demonstrated a time-dependent number of TUNEL-positive myonuclei in comparison to negative controls. TUNEL-positive nuclei were found both in damaged and normal-looking muscle fibres. Contrary, caspase 3 and 9 were detected only in damaged fibres. The expression of caspase 3 gradually increased from 3h to 7d after mastoparan administration whereas caspase 9 was only expressed at day 3. IFN-γ and TNF-α pro-inflammatory cytokines expression was significant from 3h to 3d, and so was the expression of CD68 and CD163, respectively proteins specific for resident and migrating macrophages. These events were transient, and at day 21 after mastoparan they were practically absent, myofibres were fully regenerated. Interestingly, the total number of fibres was higher in Polybia-MPII-injected TA than in matched controls, and 7.7% of apoptotic myonuclei persisted at day 21 suggesting likely that satellite cells were preserved from necrosis/apoptosis, or the preserved population was enough to regenerate the muscle fibres. It is likely that apoptosis is involved in muscle remodeling after Polybia-MPII i.m. injection to maintain fibre nucleus:cytoplasm ratio and tissue homeostasis.

Key words: cell death; macrophage; myonecrosis, skeletal muscle; tissue repair; TUNEL reaction

Introduction

Accidents by Hymenoptera (bees, wasps and ants) are frequent both in rural and urban areas. Amongst the wasps, those considered of medical importance belong to Vespoidea [1], among which the family Vespidae is the social wasp well-studied. From the Vespidae subfamilies, only Polistinae, the largest group of social wasps, has geographical distribution in tropical countries [2]. One important endemic Polistinae species typical of São Paulo State, Brazil, is *Polybia paulista*, responsible for the largest occurrence of accidents in the country. Two novel peptides from the mastoparan sub-class, the Polybia-MPI [3] and Polybia-MPII [4] were recently isolated from the *P. paulista* venom and have their sequence of amino acids determined. Polybia-MPI was shown to have antimicrobial and antitumor activity, and its anti-proliferative effect seems to be exerted by membrane pore formation being the molecule alpha-helix conformation a pre-requisite for Polybia-MPI antitumor activity [5]. Moreover it has been shown by in vitro and in vivo experiments that both, the crude venom and the Polybia-MPII, causes in mice skeletal fibres sarcolemma rupture, cytoskeleton degradation, marked sarcoplasmic reticulum/mitochondria damage and cell necrosis, besides morphological evidences of myonuclei apoptosis [6-9].

Mastoparan, a tetradecapeptide isolated from wasp venom, is a remarkable secretagogue exhibiting potent exocytotic activity in a great variety of cell types including mast cells, pancreatic islets β-cells, cardiomyocytes, platelet cells, cromaffin cells, lung alveolar type-2 cells [10-15], to increase cytosolic concentration of Ca^{2+} [16-17], to induce mitochondrial inner membrane permeability transition [18-19] and promote release of pro-apoptogenic cytochrome c from mitochondria [20]. Since Higashijima et al. [21] first demonstrated that mastoparan activates G-proteins, mainly G_0 and G_1, special efforts have been given to study the various peptide effects, confirming that the great majority of them involve G-protein signaling. Other effects attributed to mastoparan include activation of phospholipase A_2 [22] and phospholipase C [23]. Biophysical evidence has shown that the Polybia-MPII interacts with biomembranes and in contact with the phospholipids bi-layer adopts an amphipathic conformation in a “carpet” named configuration which in turn would cause the disturbance of the lipids bi-layer and membrane permeability disruption (Unpublished results)**.** In a previous study, we hypothesized that the muscle fibres damage

caused by Polybia-MPII [8] could be linked to the peptide capacity of disturbing both Ca^{2+} mobilization [24-25] and protein kinase C (PCK) function [26], given the sarcolemma mechanical stability requires PCK mediated-phosphorylation of dystrophin and its binding to myofibrils cytoskeleton, a phenomenon which is Ca^{2+}-mediated [27-28]. We also raised the hypothesis that the evidence of apoptosis would involve mitochondrial pathways, as suggested by mitochondrial damage [9]. Chapman [29] attributed to mastoparan sharing homology with the amphipathic helix forming death domains of the tumour necrosis factor receptor-1 (TNFR-1) and Fas, the cytotoxic action exhibited by the peptide. Since some of the described actions of mastoparan can explain mechanism by which cell death is mediated its use as tool to study important issues in cell biology is of great interest.

In the present study we have studied inflammatory and apoptotic cellular and molecular events occurring in the tibial anterior muscle in different time-points after intra-muscular injection of mastoparan Polybia-MPII from the *P. paulista* wasp venom.

Material and Methods

Animals

Male Balb/c mice (~25 g) were obtained from an established colony maintained by the Animal Services Unit of the State University of Campinas (CEMIB-UNICAMP) and kept in a temperature-controlled room (20 ± 3°C) with a 12 h light/dark cycle and free access to water and standard chow (Purina). All experimental protocols were approved by the University's Committee for Ethics in Animal Experimentation (CEEA/IB, Protocol 701-2), following the principles established by the Brazilian College of Animal Experimentation (COBEA).

Polybia-MPII mastoparan and experimental procedure

The Polybia-MPII mastoparan (INWLKLGKMVIDAL-NH_2) was synthesized by step-wise manual solid phase synthesis using N-9-fluorophenylmethoxy-carbonyl (Fmoc) chemistry with Novasyn TGS resin (Nova Biochem). After the role process, the crude

peptide was suspended in water and chromatographed under reversed-phase HPLC (RP-HPLC) in a semi-preparative column (Shiseido C18, 250 x 10 mm, 5 mm), with a isocratic elution of 45% (v/v) acetonitrile in water [containing 0.1% (v/v) trifluoroacetic, TFA] at a flow rate of 2 mL/min. ESI-MS analysis and Automatic Edman Degradation Chemistry was performed to check the peptide purity and sequence, and the peptide was lyophilized and stored at -20^{o}C.

Mice were deeply anesthetized with ketamine chloride (Dopalen®, Vetbrands, 100 mg/kg of animal) and xylazine chloride (10mg/kg, Anasedan®, Vetbrands) (1:1, 2 µl/mg body weight, i.p.) and the right tibialis anterior (TA) muscle was injected with intramuscular injection (100 µl) of saline solution (control groups) and 0.25 µg/µl Polybia-MPII (treated groups). After 3 and 24 h, 3, 7 and 21 d (n = 6), mice were anesthetized and TA muscles were dissected for further procedures.

TUNEL

The TUNEL (terminal deoxyribonucleotidyl transferase mediated dUTP nick end labelling) reaction was applied to analyse DNA fragmentation in control and mastoparan Polybia-MPII treated TA muscles. The detection of apoptosis by labelling 3'-OH DNA ends of single- and double-stranded DNA fragments was performed using the *In Situ* Cell Death Detection Kit (Roche Diagnostics GmbH, Germany).

Control and Polybia-MPII treated TA muscles were fixed in 4% paraformaldehyde and routinely processed until paraffin embedding. The methodology used was according to the kit's protocol. Section (5 µm thick) were dewaxed in xylene and hydrated in ethanol series (100%, 95%, 90%, 80%, 70% and distilled water), rinsed in PBS before Protease K treatment. After, the sections were permeabilized in a solution with 0.1% Triton X-100 and 0.1% sodium citrate, rinsed in PBS and incubated with TUNEL reaction mixture for 1 h. Positive controls were set up by pre-incubation with DNAse I for 10 min before the TUNEL method, while the negative control was incubated with the label solution without terminal transferase.

The slides were washed in PBS, incubated in converter-POD reagent for 30 min, and washed again before adding DAB substrate to signal conversion. Finally, the slides

were rinsed, dehydrated and mounted to light microscope analysis in an Olympus BX51 microscope (Olympus, Japan). Serial sections were stained with hematoxylin and eosin (HE). TUNEL-positive nuclei and HE-stained nuclei were counted in transversal sections of the tibial anterior muscle (n = 6 mice, 3 sections per animal, total of 18 sections/time interval). Data were expressed as TUNEL index, which was calculated dividing the TUNEL positive nuclei by the total number of HE-stained nuclei X 100. In order to evaluate possible fibre loss induced by the MP, comparison was done between the total numbers of muscle fibres in saline- and MP-injected at day 21 after the treatments.

Caspase 3 and 9 immunohistochemistry

Control and Polybia-MPII treated TA muscles were embedded in OCT-Tissue Tek and frozen in n-hexane with liquid nitrogen (-40°C) until use. Cryostat sections (10 μm thick) were collected on subbed slides, air dried and fixed in acetone at -20°C. After washing in TBS 0.05 M, the sections were permeabilized with 0.3% Triton X-100 for 10 min, rinsed again in TBS and maintained in 1% TBS/BSA at room temperature (RT) during 1 h. The sections were then incubated with primary rabbit anti-caspase 3 and anti-caspase 9 polyclonal antibodies (1:50, Sigma), diluted in 1% TBS/BSA. Sections were incubated overnight at 4°C in a humid chamber. After rinsing in 0.05 M TBS the slides were incubated in a FITC-labelled anti-rabbit and TRITC-labelled anti-rabbit secondary antibody (Sigma, 1:100 both diluted in 1% TBS/BSA), for 2 h in a humid chamber at RT. The slides were then rinsed in TBS and mounted in Vectashield (Vector Labs.) for further analysis with an Olympus BX51 microscope (Olympus, Japan).

Western blotting analysis of caspase 3 and 9

Western blotting was applied to determine caspase 3 and 9 expressions in control and Polybia-MPII injected TA muscles. Muscles were homogenised in an extraction cocktail (10 mM EDTA, 2 mM PMSF, 100 mM NaF, 10 mM Na pyrophosphate, 10 mM $NaVO_4$, 10 μL aprotinin/ml and 100 mM Tris, pH 7.4) and the samples were diluted in loading buffer (5% PBS buffer, 50% glycerol, 10% SDS, 10% β-mercaptoethanol, 10%

bromophenol blue). The homogenates were analysed by electrophoresis in a 10% polyacrylamide gel (Mini Protean 3, Bio-Rad) and western blotting was performed by loading with 30 μg proteins/lane. Nitrocellulose membrane was incubated overnight at 4°C in Tris-buffered saline (TBS, pH 7.4) containing 0.05% Tween 20 with 5% non-fat milk at RT during 2 h with primary rabbit anti-caspase 3 (1:200, Santa Cruz Biotechnology) and anti-caspase 9 (1:50, Sigma) antibodies, both diluted in a solution containing TBS and 0.1% Tween 20 and 3% non-fat milk.

The membranes were rinsed in TBS buffer and incubated with HRP-conjugated anti-rabbit secondary antibody (1:1000 and 1:250, respectively, Sigma) diluted in TBS, 0.1% Tween 20 with 1% non-fat milk for 1 h in a humid chamber at RT. After rinsing in TBS buffer, the signals were enhanced chemiluminescence kit (Super Signal, Pierce) and then visualized by exposing the membranes to X-ray films (Eastman-Kodak) and digital records were captured with a photographic paper. The resulting bands were quantified as OD x band area by one-dimensional image analysis system (Un-Scan-IT Gel software). Values were expressed in arbitrary units (AU).

Transmission electron microscopy

To illustrate the damage of mitochondria in muscle fibres of mice sacrificed after 3 and 24 h, 3, 7 and 21 days (n = 6/period) of Polybia-MPII mastoparan injection, the mice were anesthetized and the TA muscles were dissected. Briefly, the muscles were sectioned in two halves at the point of MP injection and vicinal fragments of TA were fixed and processed as described elsewhere for analysis in a Leo 902 Zeiss transmission electron microscope operated at 60 kV [9].

TNF-α, IFN-γ immunohistochemistry

Control and Polybia-MPII treated TA muscles were fixed in 4% paraformaldehyde and processed to paraffin embedding. The slides (5 μm thick sections) were dewaxed in xylene and passed in ethanol graded series (100%, 95%, 90%, 80%, 70%, and distilled water) rinsed in 0.05 M PBS and pre-treated with H_2O_2: 50% ethanol (1:1000) solution for

15 min, to quench endogenous peroxidase activity. After, the slides were rinsed in citrate buffer and maintained in a vapour pan, for 30 min.

After rinsing in 0.05 M PBS and incubation with 1% PBS/BSA for 2 h, the slides were washed and then incubated with primary rabbit anti-TNF-α and rabbit anti-IFN-γ polyclonal antibodies both diluted in 1% PBS/BSA (1:50, Peprotech) overnight at 4^{o}C in a humid chamber. After several rinses in PBS, the sections were incubated with biotinylated anti-rabbit secondary antibody (kit Santa Cruz Biotechnology). The reaction was amplified with HRP-Streptavidin and developed with DAB. Haematoxylin was used to display the nuclei morphology. Finally, the slides were rinsed, dehydrated and mounted with Canada balsam to light microscope analysis in an Olympus BX51 microscope (Olympus, Japan).

Evaluation of the immunopositive reaction was done by computer-aided measurements of the positive cells-containing area. Calculation of the relative area (%) positive for the cytokines was assessed by multiplying positive TNF-α or IFN-γ areas by 100 and dividing them by the whole tibial anterior cross-sectional area both saline- and MP-injected at each time-point scheduled (n = 6 mice/period).

Similarly, the relative regenerating area (%) was assessed by multiplying the areas containing myoblasts, myotubes and central-nucleate myofibres and positivity to TNF-α or IFN-γ by 100 and dividing them by the whole cross-sectional area of the muscle. The unaltered area was evaluated by simply subtracting the damaged and/or regenerated positively-labeled area from the whole section of the muscle. From all experimental groups three cross sections, 50 μm distant from each other, were taken, given 150 μm of tissue width.

Resident and migrating macrophages immunohistochemistry

Immunohistochemistry of CD68 glycoprotein (from resident macrophage) and CD163 protein, a member of the scavenger receptor cysteine family (restricted to the migrating macrophage lineage) were done in cryosections of saline- and Polybia-MPII-injected TA (see caspase protocol above) of 3 h and 24 h and 3 days after Polybia-MPII i.m. injection (corresponding to the peak periods of caspase-3 activity). The slides (with TA longitudinal sections, 5 μm thick) were fixed for 3 min in cold acetone, hydrated in TBS (5

min) and pre-treated with H_2O_2:TBS (1:3000) solution for 10 min. After, the slides were rinsed in TBS, incubated with 3% TBS/BSA for 2 h, re-washed and then incubated with primary ab overnight at 4°C in a humid chamber. Several rinses were done and sections were incubated with the secondary ab, at 36° C for 1 h. Both primary antibodies [monoclonal rabbit anti-CD68 – Serotech Ltd (Oxford, UK) and polyclonal rabbit anti-CD163 – Dako (CA, USA)] were used at a dilution of 1:50. The reaction was amplified with anti-rabbit HRP-Streptavidin (Dako Cytomation LSAB + System-HRP) and developed with DAB. Haematoxylin was also used to display the nuclei morphology.

Statistical Analysis

All data were presented as means ± SEM of the number of animals used or experiments done. Statistical inter-group comparisons was done using one-way analysis of variance (ANOVA) and the Tukey–Kramer multiple comparisons test, with $p<0.05$ indicating significance.

Results

TUNEL

Negative-controls did not show nuclei labelled with TUNEL, whereas the positive controls showed 100% TUNEL-positive nuclei in the TA muscle sections. As shown in Figure 1, in general the Polybia-MPII treated muscle (T) showed a time-point significant difference in the percentage of TUNEL-positive nuclei when compared with the matched control (C) muscles: 40.1% ± 10.3% *vs* 6.6% ± 2.9% after 3 h ($p<0.0001$); 24.7% ± 5.2% *vs* 5.8% ± 3.3% after 24 h ($p<0.0001$); 24.3% ± 7.9% *vs* 7.3% ± 1.8% after 3 d ($p=0.0001$); 13.1% ± 5.0% *vs* 5.0% ± 2.5% after 7 d ($p=0.0039$), except for C-21 d and T-21 d: 7.7% ± 4.5% *vs* 5.3% ± 2.9%. Differences in the proportion of apoptotic nuclei, between treated-groups were also seen, except between T-24 h and T-3 d ($p<0.0001$), (Fig.1; see also figure 4A-E). Counting of total (intact and regenerating fibres) in day 21 after saline or

mastoparan i.m. injection showed that there was an increment of 9.7% of muscle fibres in Polybia-MPII TA muscles and 7.7% of TUNEL-positive nuclei persisted at this time-point.

Caspase 3 and 9 immunohistochemistry and immunoblotting

The occurrence of apoptosis after injection of Polybia-MPII was confirmed by immunohistochemistry and immunoblotting for caspase 3 and caspase 9. The immunohistochemistry showed that both proteases were absent from intact fibres and expressed in damaged fibres at 3 and 24 h, respectively, in MP-treated muscles (Fig. 2). Western blotting of the proteases corroborated the immunohistochemical results (Fig.3A) (n =3/each). There were noticeable expression of caspase 3 and 9 in mice injected with MPII, whereas their expression in control mice was around 8%. The results showed that caspase 3 expression was significantly point-by-point higher than the expression of caspase 9, and significant differences were observed in all periods ($p<0.01$ and $p<0.001$). A bi-modal peak of activity, the first of which at 24 h and the second at day 7, was shown for both caspases. At day 21, the expression of both proteases is almost the same observed after day 3 (Fig. 3B).

Transmission electron microscopy

The ultrastructure of mitochondria of TA muscle injected with sterile saline was normal, as well was normal the topographical situation of the organelles inside the fibre. In contrast, in Polybia-MPII injected ones, fibres affected by the peptide showed displacement of mitochondria from its usual intermyofibrilar, and/or subsarcolemmal situation. In addition, mitochondria from fibres affected by the peptide exhibited marked alterations involving all the components of the respiratory organelle: cristae, inner and outer membrane and mitochondrial matrix (Fig. 4). Alterations include both the merging and electrondensity of cristae, electrolucent matrix, rupture/distortion of the inner, outer and/or cristae membranes and appearance of flocculent densities. These alterations were prevalent in degenerating fibres seen at 3 and 24 h of envenoming (were absent in intact fibres) and decrease in later periods until complete recovery.

TNF-α and IFN-γ immunohistochemistry

In the control groups, less than 2% of the tissue cross-sectional area (CSA) was immunopositive to TNF-α or IFN-γ. At each time-point of the trial period there was significant increase in the expression of both cytokines in MPII-treated TA in comparison to saline-injected muscle. In addition, Polybia-MPII-treated TA muscles showed different proportions of CSA which was immunolabelled for either TNF-α or IFN-γ along the trial period, respectively as follows: 14% ± 5.5 *vs* 19% ± 6 after 3 h; 16% ± 3 *vs* 17% ± 7 after 24 h; 28% ± 18 *vs* 18% ± 13 after 3 d; 6% ± 4 *vs* 11% ± 5 after 7 d; 2% ± 1 *vs* 2% ± 2 after 21 d (Fig. 5 and 6). Significant differences were observed in the CSA labelled for TNF-α and IFN-γ expression at 7 d and 21 d ($p>0.05$). However, whereas TNF-α-labeled CSA gradually increased until day 3 d (~30%) after which a significant shift occurred (3d > 7d, $p<0.01$ and 3d > 21d, $p<0.001$), the IFN-γ-labeled CSA remained stable from 3 h to 3 d (~18-20%) for thereafter to gradually decrease until the end of the trial period when it was reduced to about 2% (3 h = 3 d > 21d , $p<0.05$) (Fig. 6).

Both cytokines were immunoexpressed in the hypercontracted fibres, just upon the region where there was condensation of myofilaments. The peak of both cytokines paralleled with the higher number of infiltrating inflammatory cells (Fig. 5F-O). See Figures 5F-J and 5K-O for TNF-α and IFN-γ, respectively.

Resident and migrating macrophages immunohistochemistry

The results showed that both residents (expressing CD68 glycoprotein) and migrating macrophages (expressing CD163 protein from scavenger receptor cysteine family) were expressed after 3, 24 h and 3 d of envenoming with *Polybia*-MPII. Controls, saline injected TA were negative. Both proteins CD68 and CD163 containing macrophages were present just over the damaged portions of the muscle fibres. No difference was observed in the number of the two populations of macrophages in the trial period (Fig. 7).

Discussion

The mastoparan Polybia-MPII (INWLKLGKMVIDAL-NH_2) has been characterized as a myotoxic component from the *P. paulista* wasp venom capable of rapidly disrupting the plasma membrane of skeletal muscle fibres and provokes myonecrosis and fibre death [8-9]. Concomitantly, interstitial infiltrating inflammatory cells were recruited very early after the Polybia-MPII i.m. injection and were still present on day 3 from envenoming. Despite the drastic damage inflicted to a broad number of muscle fibres they were feasible to fully regenerate and no visible clinical sequel was noticed in the mice until 21 days after envenoming. In fact, one week later from Polybia-MPII administration regeneration was seen in frank progress and signs of damaged tissue were imperceptible, apart presence of split-fibres and increased cellularity, mainly represented by fibroblasts, among the regenerating fibres [9].

Interestingly, our current data showed a 9.7% rise in the proportion of myofibres in TA muscles treated with Polybia-MPII compared to the saline controls at day 21 after envenoming. In addition, 7.7% out of the exceeding 9.7% muscle fibres showed TUNEL-positive nuclei. This phase corresponds to the remodeling phase (~5-30 days) and overlaps with the regenerative phase (~2-7 days) to which muscle tissue was subjected after injury [30]. The fact mastoparan injected-TA muscles exhibited higher number of fibres than saline injected ones suggests that muscles affected by the peptide can present a relative upregulated proliferative activity. This indicates very likely that an-extra number of myofibres has regenerated, and may imply a putative expedient to prevent muscle mass shortage in case of further myofibres loss, what seems plausible since a parcel of these fibres presented TUNEL positive nuclei. As well, the higher number of apoptotic nuclei in MP-injected TA at day 21 may imply a mechanism assumed by fibres with risk of an unsuccessful survival to maintain nucleus:cytoplasm ratio and tissue homeostasis.

In the present study, both types of events, necrosis and apoptosis, were induced by Polybia-MPII. The hypothesis is that under the peptide effect cells fated to die by necrosis would amend their program of death by apoptosis and vice versa. However, because muscle fibres can reach several centimeters length, in longitudinal sections we could see that the same fibre was necrotic in an ending and showed TUNEL positive nuclei in regions where

structure was more preserved and cell membrane was continuous (not shown). Other fibres, which in full appeared normal-looking in structure with light microscope observation, showed both apoptotic nuclei and non-apoptotic ones, suggesting that multinucleate cells such as muscle fibres can show nuclear apoptosis without loss of the fibres. Our studies, shows evidence that mitochondria was involved in the apoptosis of fibres nuclei caused by MP, probably involving mitochondrial permeability transition (MPT) and cytochrome c release (18-20) and caspase signaling pathways as shown here. Also, because muscle fibres can work either in oxidative or glycolytic milieu depending on the mitochondrial content, the effect of mastoparan in inducing mitochondrial permeability transition (MPT) can vary accordingly, which could explain the variability in apoptotic nuclei number in the fibres.

On the other hand, MPT is causative of both necrotic and apoptotic cell death and can be induced by other agents than mastoparan, such as oxidative stress, Ca^{2+} ionophore toxicity and TNF-α [see 31, 32 for review]. The findings showed that reactive resident and migrant macrophages were immunodetected as early as 3 h after MP-injection, whereas saline-control TA did not show any phagocytic cells. It is likely that resident macrophages (total macrophages) within the TA muscle tissue were inactive under physiologic (saline) conditions and as such remained dormant in regard to defense activities. When subjected to Polybia-MPII insult, the resident macrophages were activated, passing to express CD68 glycoprotein and release chemo attractants and pro-inflammatory cytokines [33], in response to which inflammatory polymorphonuclear cells were recruited to the damaged area, as seen here in the early stages of mastoparan i.m. injection. Our results showed that the severity and quality of insult caused by the Polybia-MPII was able to promote also the fast arrival of migratory macrophages, born from circulating monocytes, which expressed CD163 protein, a member of the scavenger receptor cysteine family.

The recruitment of such macrophage population signalizes that muscle tissue was injured and necrotic cell debris needed to be removed from the injured site. Such a reaction suggests generation of an anti-inflammatory response, a requisite for muscle regeneration [34]. In the present study, the co-expression of the pro-inflammatory cytokines TNF-α and IFN-γ paralleled with the higher activity of resident and migrant macrophages at 3 and 24 hours and 3 days, a time interval characterized by intense proteolysis of myofibres caused by Polybia-MPII i.m. injection [8], and corresponding to the inflammation phase (~hours-7

days) [30]. This phase has been considered critical to the development of events involved in the inflammatory cascade and further recovery of tissue to a structural and functional normality.

IFN-γ has been found to influence skeletal muscle homeostasis and repair. Administration of exogenous IFN-γ appears to inhibit fibrosis and improve healing of skeletal muscle *in vivo* [35]. On cultured muscle cells, IFN-γ increases expression of major histocompatability complex (MHC) molecules, intracellular adhesion molecule-1 (ICAM-1), and monocyte chemotactic protein-1 [36-37], as well as activate macrophages and influence proliferation and differentiation of cultured myoblasts [38-39]. Endogenous production of IFN-γ was shown to be required for efficient skeletal muscle regeneration, in part, through direct effect on muscle cells [30]. Whether the higher number of fibres in TA treated with MP in comparison to saline treated ones on the 21st day of the experimental period was a direct effect caused by MP, or indirectly caused by cytokines is not known. However, the role of endogenous IFN-γ in skeletal muscle repair has not yet been determined. Our data showed that high figures of IFN-γ expression last from 3 hours to 3 days, whereas the peak of TNF-α expression was on day 3 coincident with the period of resident and migrant macrophages major presence. Studies suggest that a cross-talk can be established between IFN-γ and TNF-α, such as TNF-α can also induce the expression of IFN-γ [40] what could contribute for muscle regeneration. However, the role of TNF-α on muscle response to damage has been subject of dispute. Prolonged activation of TNF-α production has been related with muscle fibre loss [41], and delay in muscle fibre regeneration, whereas inhibition of caspase activity improved the regenerating process of fibres [42].

In agreement, inflammatory cytokines generated by tissue damage such as TNF-α are able to activate caspase 3 [43-44] and induce actin and myosin rupture and loss [45]. In contrast, evidences suggest that elevated levels of TNF-α, whether released by infiltrating inflammatory cells, or produced by the myofibres themselves [46], as for some myopathies [47], or after damage by snake venom-derived cardiotoxins [48] have been positively correlated with improved muscle regenerating activity. In the current study, TNF-α immunolabeled-fibres occupied 16% of the TA cross-sectional area at 24 h, 28% at 3 d and fell to 6% at 7 d post-MP injection, indicating that the peak of the cytokine expression

corresponded to the climax of the regenerating process, whilst its decline corresponded to the period of tissue remodeling where intense myofibres protein synthesis are underway and fibres grow.

The peak of IFN-γ and TNF-α paralleled with major presence of resident and migrant protein macrophages and periods of high caspase 3 (two peaks, one at 24 h and the other at 7 d) and 9 expressions (peak at 24 h). Taking together, the period comprised between 3 h to 7 days in which these events took place corresponds to the inflammatory, regenerative and remodeling phases of the muscle tissue [30]. However, at day 3 a shift in the expression of both caspases took place, though being reestablished on day 7 for caspase 3, whereas TUNEL positive nuclei was highest at 3 h (~40%), stationary between 24 h-3 d (~24%) and gradually decreased thereafter. The expression of the proteases was also reduced to basal level in the later periods post-envenoming. As far as it is concerned, caspase activation requires cytochrome c release from mitochondria and is one of the pathways involved in DNA fragmentation [49]. Besides, TNF-α is known to be a central mediator in the apoptotic signalling pathways and inflammatory response [50]. The pro-apoptotic effect of Polybia-MPII could be also attributed to the peptide ability to share homology with the amphipathic helix forming death domains of the tumour necrosis factor receptor-1 (TNFR-1) and Fas [29], and the ability to cause MPT and cytochrome c release from mitochondria [18-20].

The present data show a marked mitochondrial damage at 3 h and 24 h post-envenoming what parallels with the major expression of both caspases and the peak of TUNEL positive nuclei. However, it did not match with the second peak of caspase 3 expression seen at day 7. Taken together, the fluctuation in the expression of TNF-α and IFN-γ, and the precocious appearance of macrophages expressing scavenger receptor protein reflects the dynamic complexity of cellular and molecular events which takes place during muscle envenoming by mastoparan from *P. paulista* wasp venom, and which course with pro-apoptotic events caspases-mediated and mitochondria disruption [9].

Cytokines and caspases 3 and 9 were only expressed in necrotic cells, whereas nuclei undergoing apoptosis were seen both in necrotic cells (mainly at regions of the fibre where morphology looked normal) and in intact cells (including in the regenerating ones). Such finding suggests that probably same pathways can be partially shared during the

program of cell death permitting the shift to one or another type. Because muscle fibres are a syncytium, it is likely that shifting from one to another form of death may be easily done. Switching among alternative pathways to death is a relatively common finding in a number of tissues [51].

In conclusion, mastoparan, a bioactive component of *Polybia paulista* wasp venom, was able to induce a range of cellular and molecular events in skeletal muscle, including necrosis and apoptosis. Apparently, the balance between the necrosis (where damage impelled fibres to a point of no return in its death program) and apoptosis (apparently a physiologic event not leading fibres to death) was positive as Polybia-MPII induced also an over proliferation of muscle fibres, as shown by the higher number of regenerated fibres in mastoparan-treated TA compared to controls. This suggests that in a great number of fibres the repair would be promoted by unharmed satellite cells. The fact the MP was able to induce very precocious recruitment of migrant scavenger macrophages could have an important co-adjuvant role in the apparent absence of fibre death by apoptosis and in the regenerating process of fibres.

It is suggested that apoptosis could be used as a cellular adaptation tool to promote remodeling of muscle and maintain homeostasis. As we know, there was a nucleus-cytoplasm ratio that if disrupted requires re-organization, mainly if we are considering a multinucleated fibre, in a post-mitotic tissue such as the skeletal muscle. We suggest that apoptosis can have a role in regard to this issue. This study intended to provide further insight on the effects of mastoparan in skeletal muscle tissue. Given the attributes of mastoparan, the drug has been suggested as a useful tool for therapeutic strategies, mainly having as target the inner membrane of mitochondria.

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FIGURE 1

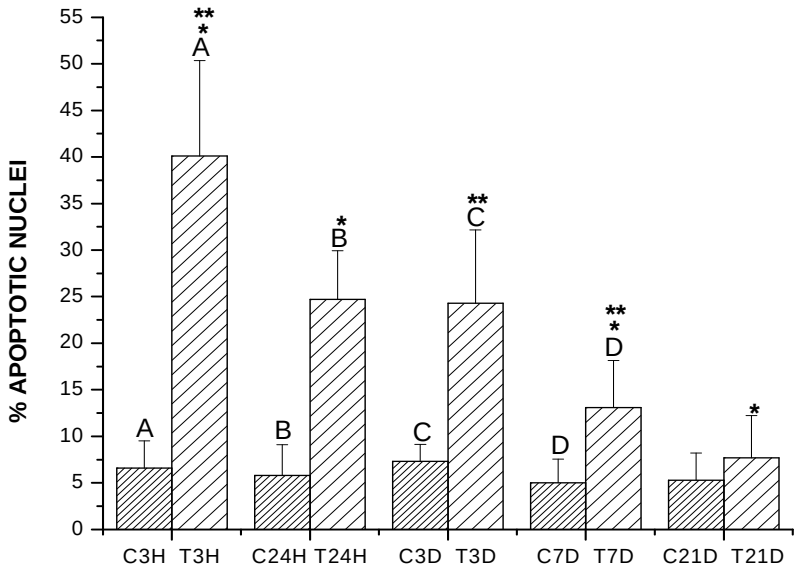


Figure 1: Average of apoptotic nuclei for each experimental group. The same capital letter **(A, B, C, D)** at each time-point indicates significant differences between MP-treated muscle and saline-treated muscle (control). (**A**, **B** - $p<0.001$; **C** - $p=0.001$, **D** - $p=0.0039$). (*) and (**) refers to significant differences in the proportion of apoptotic nuclei among MP-treated groups ($p<0.0001$). No difference was observed among control groups.

FIGURE 2

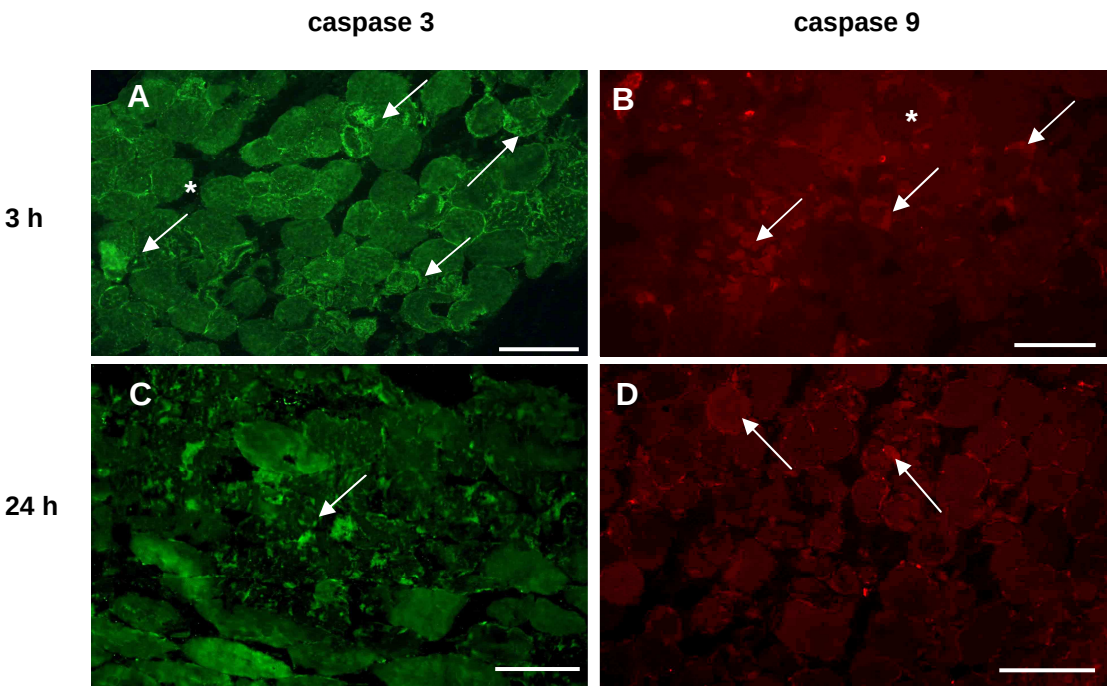
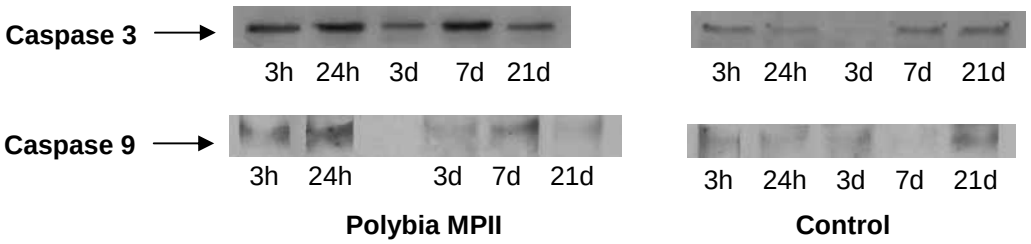
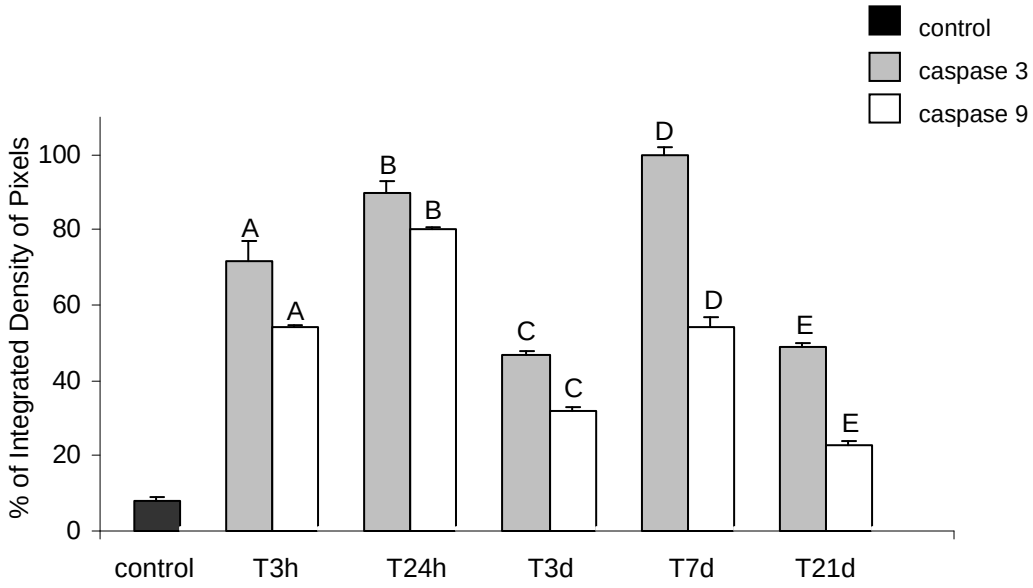


Figure 2: Transversal **(A-B, D)** and longitudinal **(C)** sections of TA muscle after 3 h **(A-B)** and 24 h **(C-D)** of Polybia-MPII injection. **A** and **C** corresponds to caspase 3; **B** and **D** corresponds to caspase 9. Note that positive reactions to both caspases were observed only in damaged fibres (arrows). No reaction was observed in normal fibres (*). Bar = 15 µm.

FIGURE 3



A: Representative blots of caspase 3 (32 kDa) and caspase 9 (~47 kDa) are shown. Note the slight expression for both caspases in the control muscles when compared with those after Polybia-MPII injection.



B: Caspase 3 and caspase 9 expressions in TA muscles after Polybia-MPII injection (treated groups). The columns represent mean ± SD of n = 3 mice per protein. Significant differences were observed between control and treated groups ($p<0.001$) and amongst treated groups: (A, C-E) ($p<0.001$), (B) ($p<0.01$).

FIGURE 4

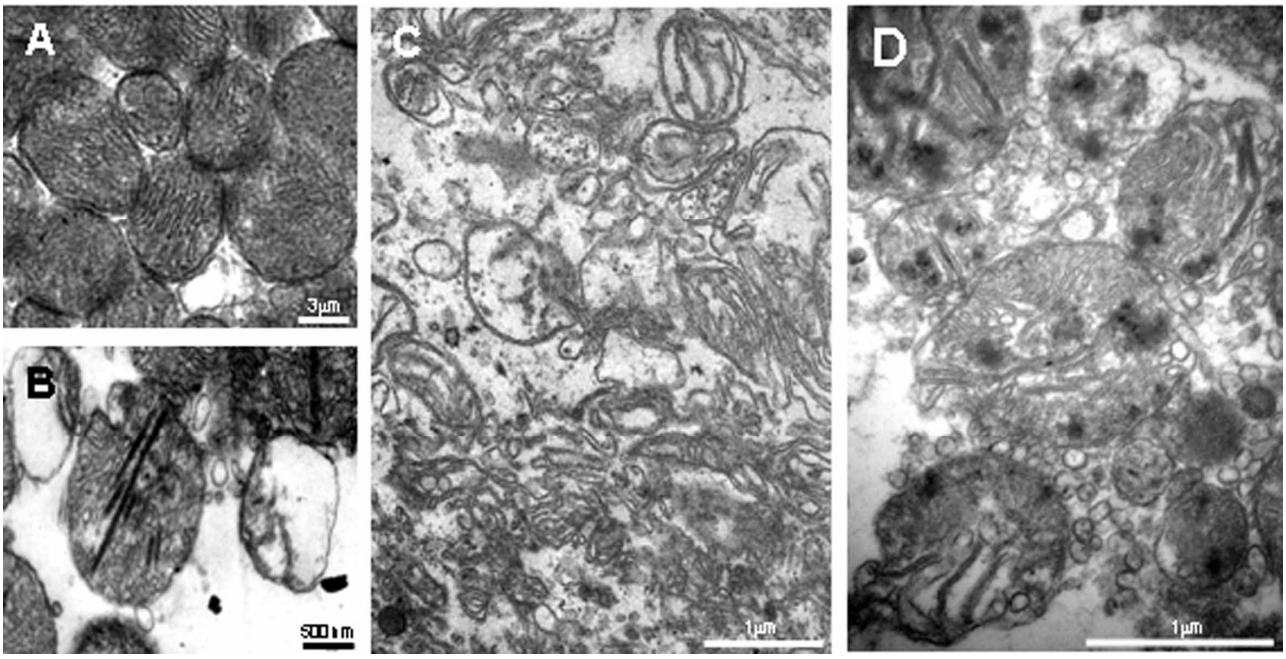


Figure 4: Electron micrographs of mitochondria in fibres of mouse tibial anterior muscle injected with saline **(A)** or mastoparan from *Polybia paulista* wasp venom at different time-points **(B-D)**. **(A)** Control: Note the normal morphology of mitochondria: intact cristae and membrane, homogeneous matrix electrondensity; **(B-C)** MP-treated 3 h, **(D)** MP-treated 24 h. MP-treated TA muscles showed mitochondria with merged electrondense and/or destroyed cristae, absence of the inner membrane and/or cristae **(B)**, free cristae scattered in the cytoplasm after different degree of mitochondrial disruption **(C),** and flocculent densities in highly abnormal mitochondria **(D)**.

FIGURE 5

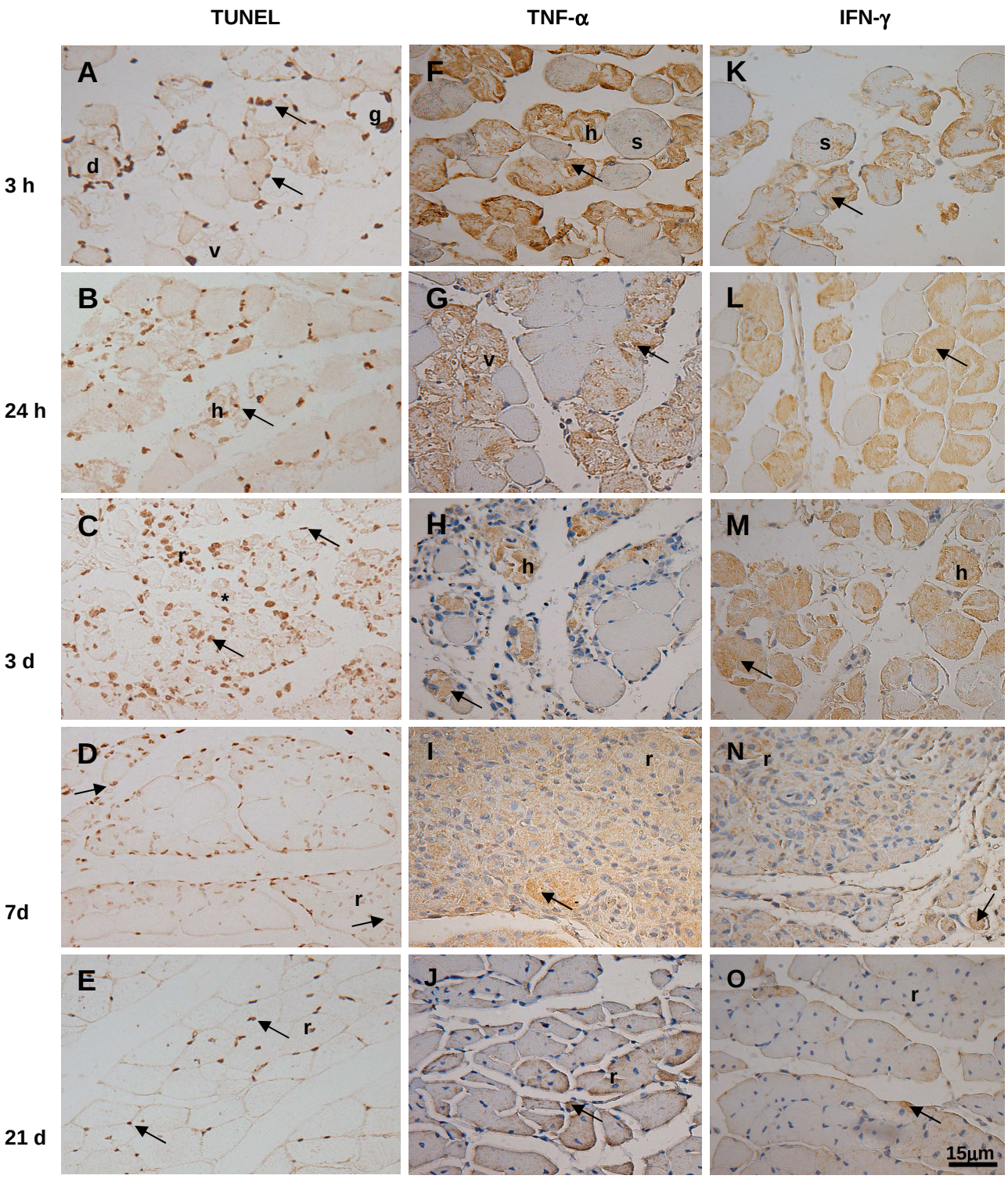


Figure 5: (A–E): Transversal sections of TA muscle at different time-points after Polybia-MPII injection and submitted to TUNEL reaction. TUNEL positive nuclei were seen in all time intervals (arrows) both in damaged fibres, with delta lesion (d), vacuolated (v), ghost fibre (g) **(A)**, or hypercontracted **(B)**, and in normal-looking (*) and including in regenerating (r) **(C-E)**. Panels **F-J** refer to TNF-a and panels **K-O** refer to IFN-g immunohistochemistry. For both cytokines immunolabeling were observed in hypercontracted fibres (arrows), but not in swollen fibres (s). At 24 h post Polybia-MPII injection vacuolated fibres showed positive labelling for both cytokines and at days 3, 7 and 21 some regenerative fibres also displayed immunolabeling.

FIGURE 6

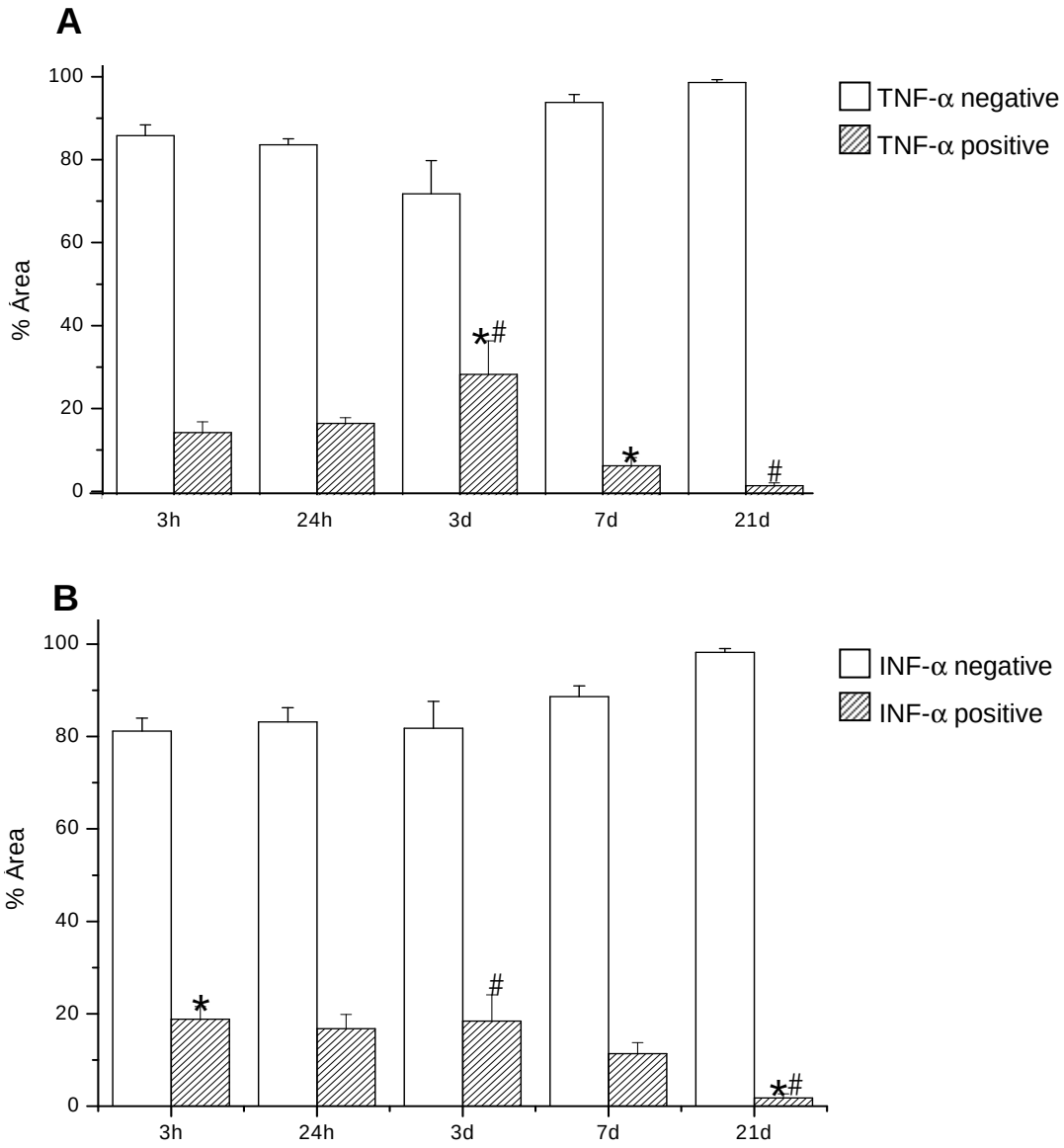


Figure 6: TNF-a **(A)** and IFN-g **(B)** expression of TA muscles after Polybia-MPII injection. The results were expressed in percentage of the cross-sectional muscle area showing positive immunolabeling for each cytokine. Significant variation of labeled area was seen when compared different time intervals as follows: **(A)** 3 d *vs* 7 d ($p<0.01$) and 3 d *vs* 21 d ($p<0.001$), **(B)** 3 h *vs* 21 d and 3 d *vs* 21 d ($p<0.05$), as indicated by * and # symbols.

FIGURE 7

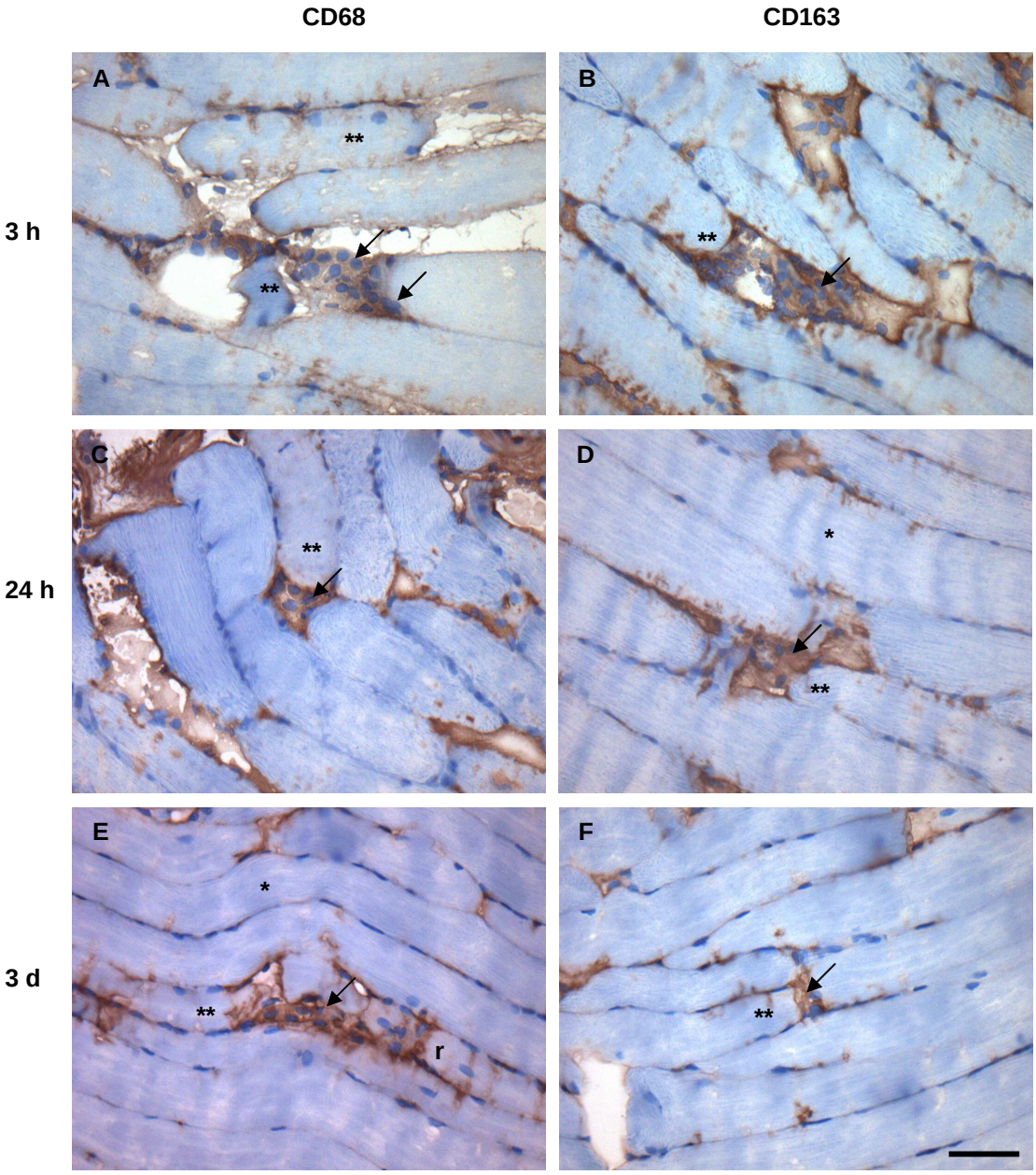


Figure 7: Immunohistochemistry for CD68 glycoprotein, specific for resident macrophages (A, C, E) and CD163 protein, a member of the scavenger receptor cysteine family, specific for migrating macrophage (B, D, F). Note the presence of both macrophage populations (arrows) close to damaged fibres (**) after 3 h (A, B), 24 h (C D) and 3 d (E, F) of Polybia-MPII mastoparan i.m. injection in TA muscle. Resident macrophages were observed on regenerative central-nucleated fibres (r) after 3 d of envenoming. Normal fibres (*) do not have any macrophage on. Bar = 20 μm.

Outros Resultados

Ação da Fosfolipase A_1 do veneno de *Polybia paulista* nas fibras musculares

Introdução

Estudos imunoquímicos de venenos de vespas mostraram que os alérgenos mais abundantes nos venenos destes insetos são as fosfolipases, hialuronidases, antígeno 5 e as fosfatases ácidas (Hoffman, 1978, King, 1980).

As fosfolipases participam diretamente na regulação do metabolismo de fosfolipídios, atuam na lise de membranas celulares, conduzem à formação de poros e levam à lise celular (Dotimas e Hider, 1987; Murakami e Kudo, 2002), e promovem a inserção dos antígenos na célula, que levam as sérias reações alérgicas e/ou inflamatórias (Nakajima, 1986).

Sua ação hidrolítica, dependente de íons Ca^{2+}, é potencializada pela atividade das lípases, esterases e fosfatases, que também atuam destruindo parte do substrato das células lisadas. A fosfolipase age sinergicamente com outras toxinas, causando lise de eritrócitos e mastócitos e podendo inibir a coagulação sanguínea (Nakajima, 1986). Em venenos ofídicos, as fosfolipases levam à permeabilização da membrana plasmática, que perde sua capacidade reguladora, levando à formação de edema celular (Lomonte et al, 1993).

As fosfolipases encontradas em venenos de vespas podem atuar aumentando a permeabilidade da membrana plasmática ao Na^{+}, resultando também na formação de edema (Rivers et al, 2002), reações alérgicas e/ou sistêmicas (Hoffman e Shipman, 1976), além de causar hipotensão, aumento da permeabilidade vascular (Reisman et al, 1987), contrações musculares e paradas respiratórias (Nair et al, 1976).

As Polibitoxinas -I, -II, -III e -IV do veneno da vespa *Polybia paulista* são fosfolipases A_2 com potente atividade hemolítica (Oliveira e Palma, 1998), além de apresentarem ação miotóxica em músculos esqueléticos de camundongos (Oliveira, 2000). Já as fosfolipases A_1, descritas por Santos e colaboradores (2007) ainda não foram caracterizadas quanto a sua miotoxicidade.

Sabe-se que as fosfolipases A_2 atuam na lise celular, e juntamente com a fosfolipases A_1 promovem a difusão do veneno nos tecidos das vítimas. Após a ativação de proteínas quimiotáticas, que desencadeiam as respostas inflamatórias e imunológicas na vítima, as fosfolipases A_1 promovem as reações alérgicas, que podem desencadear severos quadros clínicos nas vítimas sensibilizadas pelo veneno da vespa. As fosfolipases A_1 podem ser ativadas por peptídeos (mastoparanos) e induzir a liberação de mediadores associados a células TH2, mastócitos, macrófagos e possivelmente outras células apresentadoras de antígenos (Aoki et al, 2002, King et al, 2003).

Objetivos

Após a administração da fosfolipase A_1 do veneno de *P. paulista* no músculo tibial anterior de camundongos Balb/c, pretendeu-se estudar a ação desta proteína na patogênese do tecido muscular, compreendendo a fase degenerativa (3 e 24 h após o envenenamento, correspondendo aos estágios inicial e intermediário) e a regenerativa (3 e 7dias após o envenenamento, correspondendo ao estágio tardio do processo), por meio da análise morfológica em microscopia óptica.

Material e Métodos

Após a administração da fosfolipase A_1 os músculos foram coletados e fixados em paraformaldeído 4% em tampão fosfato (pH 7.4), "over night", e posteriormente desidratados em série crescente de etanol; 50% (1h), 70% ("overnight"), 80% e 95% (15 min.) e em 3 séries de etanol 100% (15 min. cada). Em seguida o material foi diafanizado em etanol + xilol (10 min) e xilol 100% (15 min) e infiltrado em xilol + parafina (15 min) para inclusão em parafina pura (1h).

As secções histológicas (5 μm) obtidas foram desparafinizadas, hidratadas e coradas com Hematoxilina-Eosina (HE). Para a coloração com HE as lâminas foram primeiramente coradas com Hematoxilina de Erlich, por 5 min, diferenciadas em água de torneira, por 5 min, e posteriormente coradas com Eosina, por 5 min. As lâminas foram lavadas em água de torneira (5 min), desidratadas, montadas com Bálsamo do Canadá sintético e analisadas em microscópio óptico Olympus BX51-PH-III.

Resultados

A fosfolipase A_1 (PLA_1) foi purificada do veneno de *Polybia paulista* utilizando-se a técnica de exclusão molecular e cromatografia de troca catiônica. A seqüência de aminoácidos foi determinada por química degradativa de Edman e comparada com a fosfolipase A_1 de outros venenos de vespas (Santos et al, 2007).

Os animais dos grupos controle e tratado não apresentaram salivação, lacrimação, coriza ou paralisia do membro injetado. Na fase inicial da mionecrose, após 3 e 24 horas de tratamento com 0,25μg/μL (i.m.) da PLA_1 do veneno de *P. paulista*, as fibras musculares afetadas apresentaram lesões do tipo delta e hipercontração dos miofilamentos, porém não foram observados vacúolos (Fig.1A-D). Embora o processo mionecrótico tenha sido atenuado, quando comparado ao observado com o mastoparano Polybia-MPII, o processo inflamatório estabeleceu-se de forma aguda já na fase

intermediária (24 h) e manteve-se em curso até o estágio tardio de 7 dias (Fig.1C-F), sem que houvesse hemorragia.

Após 3 dias (início da fase regenerativa) foram observados mioblastos e células satélites, mesmo com um intenso processo inflamatório em curso (Fig.1F). Além dos mioblastos, “split-fibres” e fibras regenerativas também foram observadas após 7 dias de tratamento, entretanto maiores e algumas já com núcleo periférico (Fig.1G-H).

Com base nestes resultados pode-se afirmar que a PLA_1 do veneno de *P. paulista* apresenta atividade miotóxica durante os estágios inicial (3 h) e intermediário (24 h) de degeneração do músculo esquelético tibial anterior e que as alterações morfológicas observadas ilustram o processo mionecrótico já caracterizado na literatura para venenos ofídicos e/ou frações de venenos (Serafim et al, 2002; Harris et al, 2003; Prianti Jr et al, 2003; Reali et al, 2003; Toyama et al, 2003; Beghini et al, 2004; Rodrigues-Simioni et al, 2004), como também para o veneno bruto de *P. paulista* e para o mastoparano Polybia-MPII. A presença de um intenso processo inflamatório poderia desencadear a síntese de citocinas que contribuiriam para o avançado processo regenerativo observado após 7 dias.

Os resultados obtidos para o mastoparano e para a fosfolipase A_1 indicam que ambos são responsáveis por promover a inflamação característica das ferroadas de Hymenoptera (Habermann, 1972; Nakajima, 1986; de Souza, 2002; de Souza et al, 2004). Porém o processo inflamatório se dá de forma mais aguda no tratamento com a fosfolipase, uma vez que estas proteínas são alergênicas (Hoffman e Wood, 1984; Nakajima, 1986).

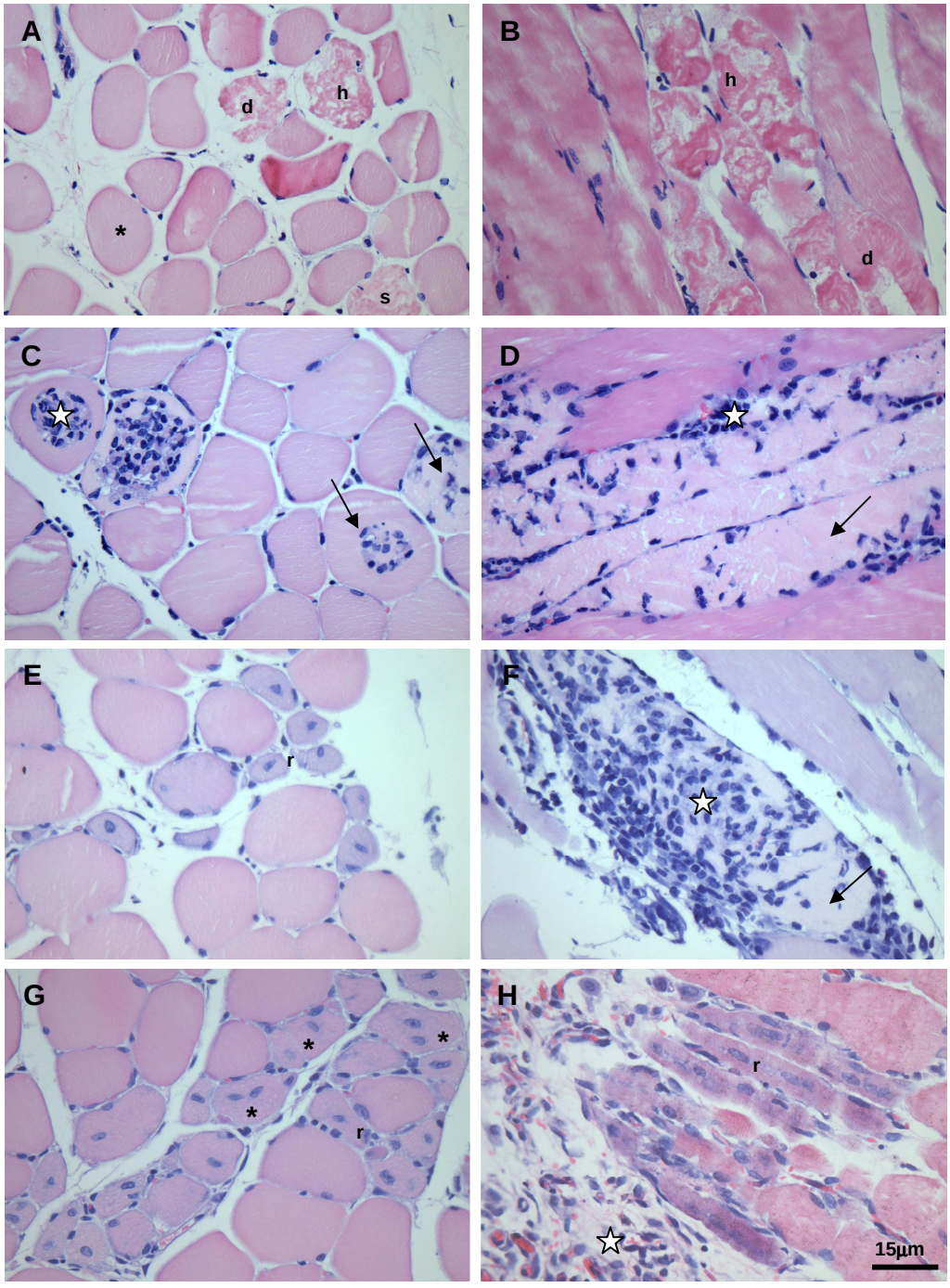


Figura 1: Músculo tibial anterior de camundogo (Balb/c) tratado com 0,25mg/ml de fosfolipase PLA1. Notar após 3 h **(A-B)** e 24 h **(C-D)** a presença de fibras mionecróticas (seta), com lesão delta (d) e hipercontração dos miofilamentos (h), entre as fibras inalteradas (*****) e o intenso infiltrado inflamatório no interior das fibras musculares alteradas ou próximo a elas (estrela). Após 3 dias **(E-F)** e 7 dias **(G-H)** fibras

Ação do Mastoparano Polybia-MPII nas proteínas estruturais da fibra muscular

Introdução

A patogênese da mionecrose, causada por diversos agentes miotóxicos encontrados em venenos animais, não só de Hymenoptera, tende a seguir um padrão comum, cujas características, nos períodos tardios, são semelhantes, independentemente de terem sido desencadeadas por toxinas hemorrágicas ou miolíticas (Ownby e Colberg, 1988).

Estas alterações mionecróticas envolvem a degeneração da fibra muscular; hipercontração de sarcômeros, perda do alinhamento das miofibrilas, dissolução de miofilamentos no citosol, permeabilização ou lise do sarcolema (Harris et al, 2003), lesão delta, condensação das miofibrilas e morte celular por necrose (Serafim et al, 2002; Prianti Jr. et al, 2003; Reali et al, 2003; Toyama et al, 2003; Beghini et al, 2004; Rodrigues-Simioni et al, 2004). Tais alterações foram observadas para o veneno bruto de *Polybia paulista* (Paes-Oliveira et al, 1998; 2000) e em músculo tibial anterior de camundongo Balb/c, após injeção i.m. do mastoparano Polybia-MPII, como descritos por Rocha et al (2007; 2008). Basicamente o que se observa após a ação de tais miotoxinas e a desestruturação das unidades contrátil da fibra muscular.

Os sarcômeros apresentam, além dos filamentos grossos (miosina) e finos (actina), um filamento composto por uma proteína denominada titina (Granzier e Labeit, 2004).

A titina (3-4 MDa) é uma das proteínas mais abundante no tecido muscular e também a mais importante entre as miofibrilas, garantindo elasticidade e estabilidade à fibra muscular, além de formar uma ancoragem com múltiplos sítios para proteínas sarcoméricas ou sinalizadoras (Liversage et al, 2001; Peng et al, 2005). Fibras musculares que não apresentam a região quinase da titina não mantêm a aparência estriada fato que atribui à titina função na manutenção da estriação da fibra muscular esquelética e cardíaca (Peng et al, 2005). Uma simples molécula de titina divide o sarcômero ao meio e interagem com ambos os filamentos grossos opostos e a linha Z. Na banda A, a titina está ligada ao filamento grosso e pode atuar como um controlador do alongamento dos filamentos, além de centralizar a banda A no sarcômero (Liversage et al, 2001).

A titina é rapidamente destruída quando o músculo é exposto a miotoxinas ou enzimas proteolíticas. Entretanto não se sabe se os processos degenerativos iniciam a desorganização da proteína ou se a degeneração é iniciada pelo rompimento do citoesqueleto (Liversage et al, 2001; Peng et al, 2005).

Já distrofina é uma das maiores proteínas (~427 kDa) musculares da família das α-actinina/espectrina/distrofina; proteínas presentes no citoesqueleto. É uma proteína flexível e alongada,

e está localizada no sarcolema. Uma série de proteínas está associada à distrofina, sendo estas proteínas periféricas da membrana, glicoproteínas sarcolemais, proteínas transmembrana, proteoglicanas extracelulares (α-distroglicana) (Earnest et al, 1995). Todas estas juntas formam um complexo citoesqueleto-matriz-transmembrana, denominado complexo distrofina-glicoproteína (Belkin e Burridge, 1995).

Embora muitos estudos tenham revelado a estrutura e as interações da distrofina, pouco se sabe sobre o mecanismo no qual sua ausência leva à mionecrose. Sabe-se que sua ausência diminui a estabilidade do sarcolema, aumentando a concentração de Ca^{2+} e levando a ativação de proteases Ca^{2+} dependentes, como a calpaina, sugerindo que a distrofina pode atuar na estabilização do sarcolema e na regulação da atividade dos canais de Ca^{2+} (Earnest et al, 1995).

Objetivos

Após a administração do mastoparano Polybia-MPII no músculo tibial anterior de camundongos Balb/c, pretende-se investigar o efeito do peptídeo nas proteínas estruturais distrofina e titina durante a fase degenerativa (3 e 24 horas) e regenerativa (3, 7 e 21 dias), por meio da análise morfológica em microscopia óptica de fluorescência.

Material e Métodos

Imunofluorescência: distrofina e titina

Os criocortes obtidos dos músculos (tibial anterior) já tratados com mastoparano Polybia-MPII (INWLKLGKMVIDAL-NH_2) foram pré-incubados em Triton X-100 0,3%, por 10 minutos, lavados em TBS 0,05M e então incubados em solução bloqueadora (TBS/BSA1%), por 1 h. Passado o tempo de bloqueio, os criocortes foram incubados "over night" com anticorpo primário (1:50), à 4ºC, sendo estes anti titina (produzido em coelho) e anti distrofina (produzido em cabra), ambos Santa Cruz Inc.

Após a incubação com o anticorpo primário, o material foi lavado (3 banhos de 5min. cada) e novamente incubado por 1 h em anticorpo secundário (1:1000), diluído em TBS/BSA1%, anti-coelho IgG conjugado com fluorocromo TRITC e anti-cabra IgG conjugado com fluorocromo FITC, ambos Sigma. Os criocortes foram novamente lavados em TBS-T e as lâminas montadas em meio aquoso Vectashield (Vector Labotratories) para análise em microscopia de fluorescência Olympus BX51-PH-III.

Resultados

Imunofluorescência: distrofina

Estágios inicial (3 h) e intermediário (24 h):

Nas fibras musculares dos animais controles, a distrofina apresentou-se imunomarcada junto ao sarcolema, como uma linha contínua e bem delineada (Fig.2A). No início do processo mionecrótico o padrão de distribuição da distrofina era mais intenso e a marcação difusa e espessa, embora se mantenha nas imediações do sarcolema (Fig.2B).

Já nas fibras nas quais o processo patológico apresentou-se mais adiantado, como lesão tipo delta e hipercontração das miofibrilas, a distrofina concentrou-se nas áreas desprovidas de miofilamentos, sugerindo a aglutinação da proteína provavelmente devido à lise do sarcolema que lhe servia de ancoragem (Fig.2C-F). Nesses estágios patológicos a expressão negativa da distrofina no contorno da fibra pode ser evidência de ausência de sarcolema, indo a proteína se alojar no interior das fibras.

Estágios tardios (3, 7 e 21 dias):

Aos 3 dias inicia-se o processo de regeneração (Fig.2G), progredindo até 21 dias quando fibras já regeneradas e com núcleo central são observadas (Fig.2H). Em ambos os estágios a expressão da distrofina se dá ao longo fibra, no sarcolema, como esperado.

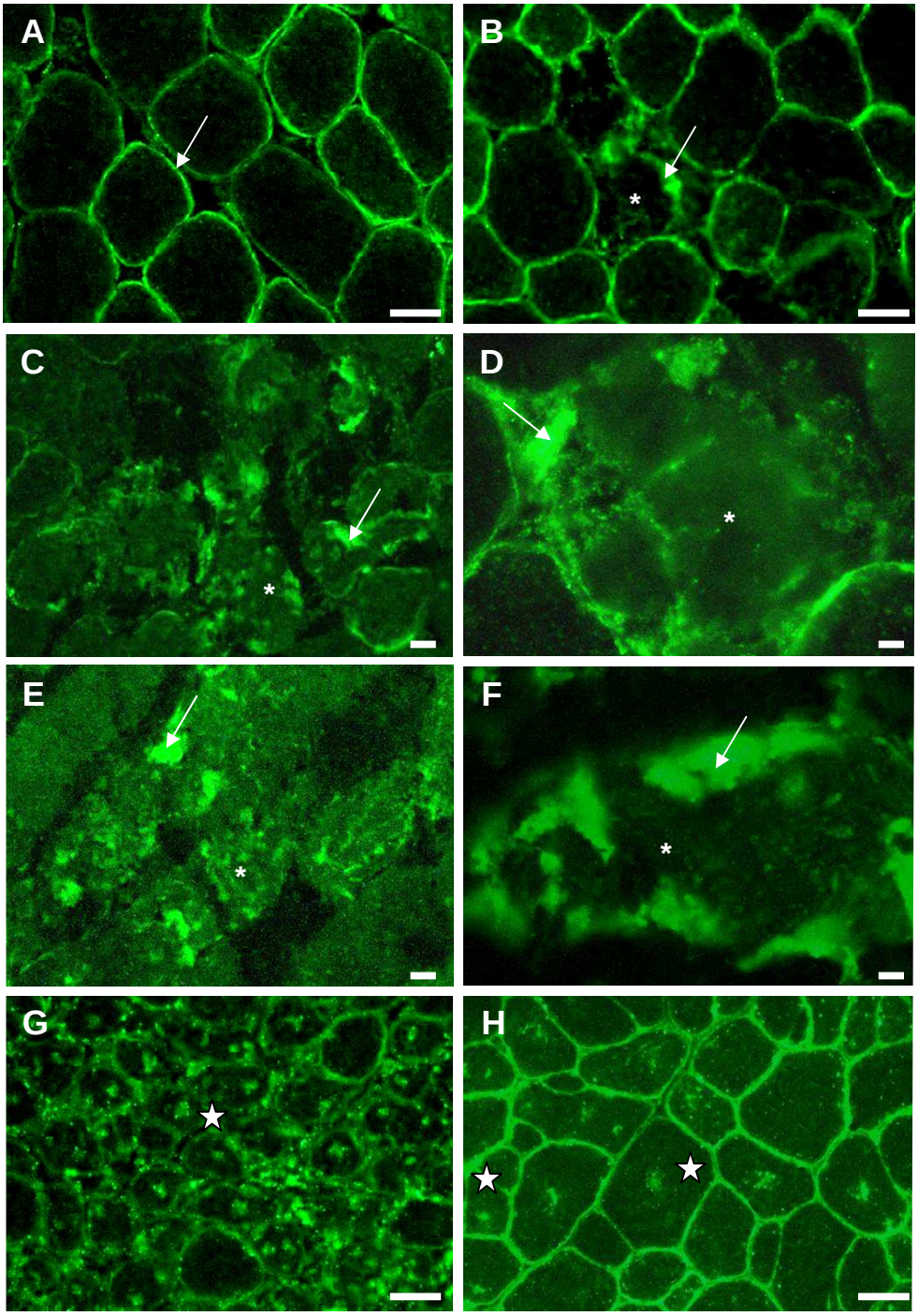


Figura 2: Criocortes do músculo tibial anterior de camundongos Balb/c. **A)** Controle. **(B-H)** Mastoparano Polybia-MPII 0,25µg/µL (i.m.) **B-C, E)** Vista geral do processo mionecrótico. Observar as fibras em lesões delta **(B)** e as fibras em hipercontração **(C)**. **D-F)** Detalhe da figura anterior indicando as fibras em hipercontração (*****); corte transversal e longitudinal. **(B-F)** 3 e 24 h. **(G)** Vista geral do início do processo

Imunofluorescência: titina

Controle: As fibras musculares dos camundongos injetados com salina mostram em corte transversal marcação puntiforme no interior da fibra (Fig.3A).

Estágios inicial (3 h) e intermediário (24 h): A imunomarcação da titina nos animais injetados (i.m.) com o mastoparano Polybia-MPII apresentou-se em forma de massas amorfas localizadas sobre a região de condensação dos miofilamentos (Fig.3B-E).

Estágios tardios (3, 7 e 21 dias):

Aos 3 d as fibras em regeneração apresentaram marcação difusa e puntiforme em seu interior, bem como próxima ao sarcolema, sendo o mesmo observado após 21 d (Fig.3F).

Quando comparados os resultados observados na imunohistoquímica para detecção de distrofina e titina aos resultados observados ao MET (ver capítulo 2), verifica-se que a forte marcação no sarcolema sugere que as fibras mionecróticas não perdem as proteínas, contrariando o observado para outras miotoxinas (Liversage et al, 2001; Peng et al, 2005), porém sofrem uma redistribuição das mesmas no sarcolema e no sarcoplasma, como relatado nos casos de doenças cardíacas crônicas (Granzier e Labeit, 2004), sugerindo que a redistribuição dessas proteínas pode explicar o desagregamento dos sarcômeros e a liberação destas proteínas no sarcoplasma.

As fibras inalteradas e as fibras regeneradas também apresentaram marcação positiva ao longo do sarcolema para ambas as proteínas (Granger e Lazarides, 1978; Debus et al, 1983; Wang e Ramirez-Mitchell, 1983; Liversage et al, 2001; Peng et al, 2005).

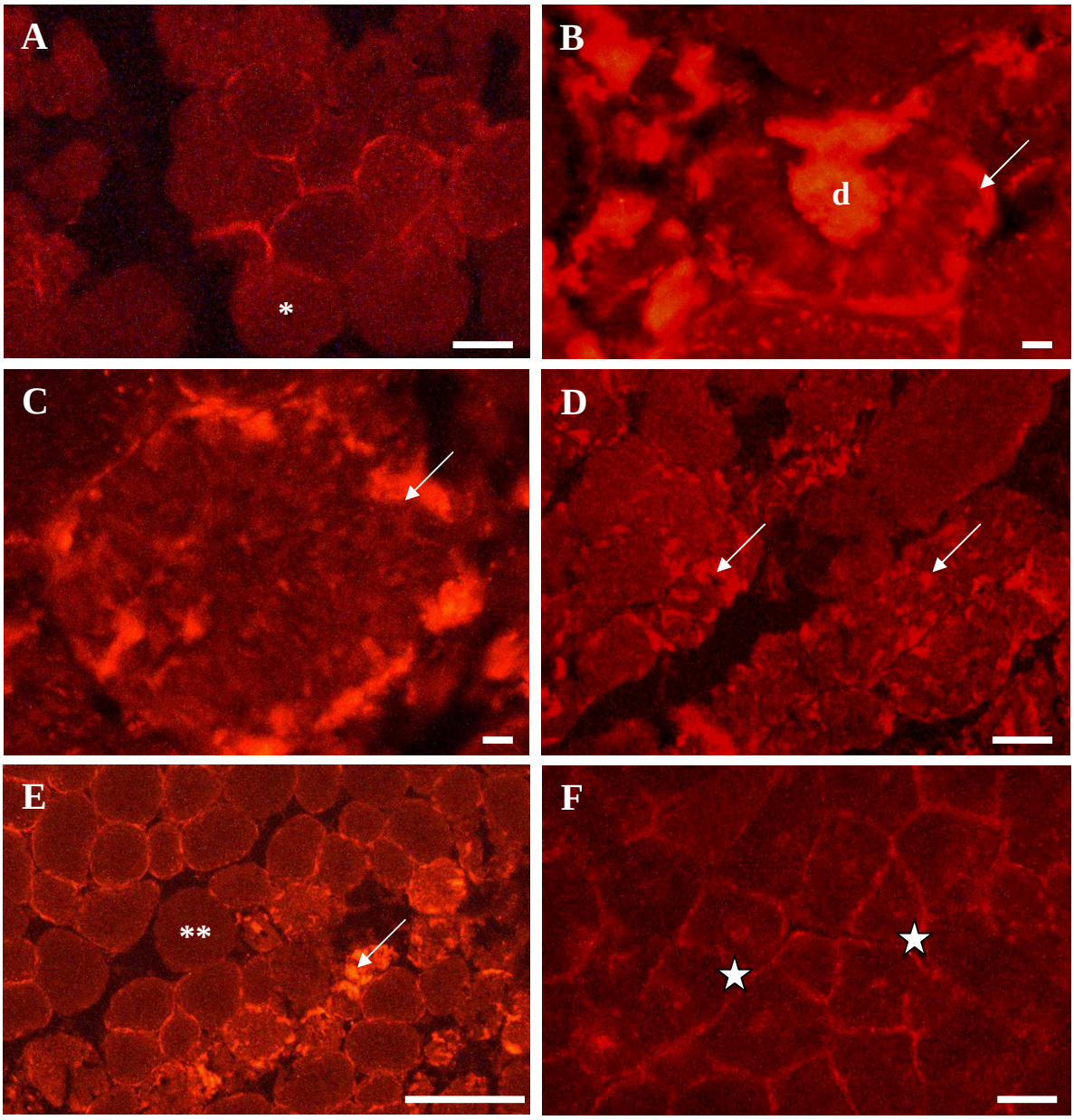


Figura 3: Criocortes do músculo tibial anterior de camundongos Balb/c. **A)** Controle. **B-F)** Mastoparano Polybia-MPII 0,25µg/µL (i.m.). **B-E)** Vista geral do processo mionecrótico após 3 e 24 h. Fibras inalteradas (*), fibras em hipercontração (seta). **E)** Fibras sem marcação puntiforme (**). **(F)**. Notar as fibras regenerativas e seus núcleos centrais (estrela) após 21 dias. Barra = 15 µm

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Conclusões

1. Os resultados mostram que o peptídeo Polybia-MPII é uma potente miotoxina, atuando principalmente no sarcolema, bem como em outras estruturas membranosas como as cisternas do retículo sarcoplasmático (RS), os túbulos T, e as mitocôndrias, além de possivelmente atuar, direta ou indiretamente, na distrofina e na mobilização de Ca^{2+}. A hipótese é que estes efeitos ocorrem por inibição de diferentes quinases e pelo aumento e diminuição da concentração de Ca^{2+}, sendo este possivelmente recapturado pelo RS. Alterações na homeostase do Ca^{2+} podem promover a permeabilidade das mitocôndrias, a perda de íons e eventualmente ocasionar morte celular por necrose e apoptose. A natureza anfifílica do Polybia-MPII promove um efeito "detergente" sobre a membrana.

2. Ultraestruturalmente pode-se observar que o mastoparano Polybia-MPII promove o dano no sarcolema, sem destruir a lâmina basal. A organização dos sarcômeros é completamente perdida, porém não há aparente dano nas células satélites, o que pode ser constatado uma vez que o processo regenerativo inicia-se rapidamente após 3 dias e aos 21 dias apresenta-se bem adiantado, sugerindo que a toxina não afeta as células satélites.

3. Os resultados obtidos indicam que ambos os eventos, necrose e apoptose, foram induzidos pelo mastoparano Polybia-MPII, que é capaz de promover o aparecimento de macrófagos migrantes diretamente envolvidos nos processos de necrose. A presença de núcleos TUNEL positivos, bem como a expressão de caspase 3 e 9 nas fibras musculares afetadas comprovam a ocorrência de apoptose após i.m. do mastoparano *P. paulista*. A presença de TNF-α e IFN-γ confirmam o processo inflamatório em curso e também, após 3, 7 e 21 dias, indicando a participação de tais citocinas no início do processo regenerativo. A co-expressão das citocinas pró-inflamatórias, TNF-α e IFN-γ, e a alta atividade dos macrófagos residentes e migrantes, são observadas durante o processo de degeneração (após 3 e 24 horas) e correspondem a fase inflamatória (~horas-7 dias).

4. O mastoparano Polybia-MPII também pode ser considerado uma neurotoxina uma vez que atua na junção neuromuscular (JNM). A redução da área seccional transversa dos botões nervosos e a diminuição do volume das vesículas sinápticas nos mesmos são indicativas da ação do peptídeo na degradação do botão nervoso. Porém não foram observadas alterações na estrutura da JNM e tão pouco nos neurofilamentos.

5. A presença do triptofano na molécula de Polybia-MPII auxilia sua orientação de forma a proporcionar uma perfeita orientação dos resíduos hidrofílicos e hidrofóbicos do peptídeo à membrana plasmática. Sugerindo que o mastoparano Polybia-MPII forma um "carpete" de moléculas sobre o sarcolema, ancorado pelo triptofano, promovendo a ruptura dos lipídeos, o desequilíbrio iônico e a abertura de canais e poros na membrana, podendo levar a despolarização da fibra muscular e ao aumento do Ca^{2+} intracelular.

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Doutorado** intitulada "Ação do Mastoparano *Polybia*-MPII nas fibras musculares e na junção neuromuscular: Um estudo morfológico, imunohistoquímico e biofisico".

(X) não se enquadra no Artigo 1°, § 3° da Informação CCPG 002/06, referente a bioética e biossegurança.

( ) está inserido no Projeto CIBio (Protocolo nº _____), intitulado _____

(X) tem autorização da Comissão de Ética em Experimentação Animal (Protocolo nº 701-2). Aprov 9/9/2004

( ) tem autorização do Comitê de Ética para Pesquisa com Seres Humanos (Protocolo nº _____).

_____
Aluno(a): Thalita Rocha

_____
Orientador(a): Maria Alice da Cruz-Höfling

Para uso da Comissão ou Comitê pertinente:

(X) Deferido ( ) Indeferido

_____
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CEEA/IB - UNICAMP